BACTERIOLOGICAL REVIEWS, Dec. 1973, p. 409-452
Vol. 37, No. 4

Printed in U.S.A.

Reduction of Nitrogenous Oxides by Microorganisms

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INTRODUCTION

Like green plants, a number of blue-green algae, bacteria, and fungi reduce nitrate to ammonia which is assimilated as a source of nitrogen for biosynthesis. Nitrite and hydroxylamine are intermediate products of reduction (225, 226). Nitrate may also serve as terminal electron acceptor in the anaerobic respiration carried out by a variety of bacteria that reduce the nitrate nitrogen no further than nitrite. In addition, a limited number of bacteria can reduce nitrate to elemental nitrogen by a series of anaerobic respiratory processes that, in sum, are called denitrification. In several studies, nitrite, nitric oxide, and nitrous oxide have been identified as intermediate products (12, 166, 224, 225), although nitrous oxide rather than nitrogen is the terminal product of denitrification in a few bacterial species and strains (110, 124, 285).

Mechanisms for control of the production and activity of the assimilatory and respiratory types of enzymes differ markedly. Unlike the dissimilative sulfate reducers, which have no other growth-supporting respiratory mechanisms, the dissimilatory nitrate reducers are preferentially aerobic bacteria. Constitutive nitrate respiration has not been observed in wild-type bacteria, and initiation of the synthesis of respiratory nitrate-reducing systems occurs in capable species only in cells incubated anaerobically or at low oxygen tension. Moreover, continuing anoxia or diminished oxygen tension is required for functioning after the dissimilatory systems are formed (266, 369). Significantly, however, neither synthesis nor functioning is influenced by the presence of ammonia or reduced nitrogen-containing organic metabolites.

In contrast, although a limited number of bacterial species constitutively produce the assimilatory enzymes, both synthesis and functioning of the nitrate reducing systems in nonconstitutive assimilatory bacteria are repressed or suppressed (as they are in algae and fungi) by the presence of ammonia or reduced nitrogenous organic metabolites in the culture medium or growth environment. Finally, a normal atmospheric oxygen tension either does not adversely affect the assimilatory enzymes or is less toxic to the assimilatory than to the dissimilatory systems.

Recently, the scope of interest in microbial reduction of nitrate and other nitrogenous oxides has broadened and now encompasses the following old and new areas of investigation: (i) testing new applications and improved methods for colorimetric assay for one-step reduction of nitrate to nitrite in cultures as an aid to taxonomic diagnosis (391); (ii) determination of mechanisms that control synthesis and functioning of the reductive enzymes (254, 261); (iii) identification of gaseous intermediates (12, 202, 285); (iv) identification of electron donors and organic and inorganic components of the electron transport chains linked to reduction (27, 161, 295, 305); (v) identification, isolation, and purification of enzymes and cytochromes involved in the various reductive steps (41, 51, 84, 85); and (vi) estimation of the agricultural and ecological impact of nitrate reduction on biological interactions in soil, sewage, and water (30, 36, 61, 100, 204).

Progress in studies of several aspects of nitrogenous oxide reduction was impeded until recent years by lack of a workably long-lived radioisotope of nitrogen for use as a tracer and by lack of precise and sensitive methods of analysis for gaseous intermediates. But, application of modern analytical methods such as mass spectrometry of products of ^{15}N-labeled nitrate reduction (115), gas chromatography for volatile intermediates and products (12, 202, 284), and electron paramagnetic resonance (EPR) assays for signals generated by metal- and heme-related reactions (51, 85) have increased the degree of our understanding (although much is yet to be learned). As a result of both the application of improved analytical procedures and the use of mutants blocked at various loci controlling the synthesis of reductases (and furthermore, as a consequence of increased awareness of the various interrelated influences of nitrate, nitrite, and ammonia in soil and aquatic environments), additional significance may now be attached to studies of reduction of the nitrogenous oxides. An impressive volume of information has been generated since publication of earlier reviews on nitrate and nitrite reduction (166, 224–226), and an up-dated review of this topic thus seems justified.

ASSIMILATORY REDUCTION

Bacteria

Distribution Among the Genera. The extent to which the capacity for assimilatory nitrate reduction is distributed among bacterial species is not known with certainty. The capacity for assimilation has not been systematically sought in many studies of nitrogen nutrition. It is not difficult to overlook the property if its significance is not appreciated; but at times it may come to light unexpectedly (e.g., Taylor and Hoare [354] isolated a facultative *Thiobacillus*

species that grows heterotrophically, but not autotrophically, when supplied with nitrate as sole nitrogen source). Search of a centralized listing (24) reveals only a limited number of genera of chemosynthetic bacteria that include species capable, by cultural evidence, of the assimilation of nitrate nitrogen while growing in minimal culture media (Table 1). But, additional indications are occasionally encountered in the literature. For example, *Rhizobium japonicum* grows in a glycerol-arabinose-mineral salts medium (188), and *Azotobacter* species grow in a sucrose-mineral salts medium (109), with nitrate serving as sole nitrogen source for both. The presence of nitrate represses nitrogenase synthesis in a manner similar to ammonia in the *Azotobacter* species that can reduce nitrate (70). In a mutant that does not reduce nitrate, the activity, but not the synthesis, of nitrogenase is inhibited by nitrate (319).

The genus *Clostridium* contains a number of the species that reduce nitrate to nitrite (and thence to ammonia) in complex culture media and assimilate the ammonia (24). Apparently, synthesis of nitrate reductase by these anaerobes is not regulated by the presence of reduced nitrogenous compounds as in other bacteria and fungi, but may be constitutive as in *Micrococcus denitrificans* and *Pseudomonas putida* (261). Now that Pichinoty has developed methods for distinguishing various types of nitrate reductases, perhaps reports of a greater number of species with the assimilatory capability may be expected.

Pichinoty's Type B Nitrate Reductase. Discovery. It is reasonable to assume that, because the development of enzymes necessary for assimilation of nitrate nitrogen may occur during either aerobic or anaerobic growth of many bacteria (whereas growth linked to respiratory reduction of nitrate takes place only under anaerobic conditions or those of significantly lowered oxygen tension), characteristics of the enzymes involved in the two processes are likely to differ in several respects. Pichinoty (255) contributed a means of studying some of these differences and answered several questions; but a number of others are yet to be clarified. He noted at the outset that *M.*

TABLE 1. *Genera of chemosynthetic microorganisms containing species reported to assimilate nitrate nitrogen*

$(NO_3^- \rightarrow NO_2^- \xrightarrow{?} [X] \rightarrow NH_2OH \rightarrow NH_3 \rightarrow$ amino acids)

Bacteria	Filamentous fungi[a]	Yeasts[b]
Aeromonas (263)[c]	*Actinomucor* (238)	*Brettanomyces*
Agrobacterium (24)	*Alternaria* (238)	*Bullera*
Arthrobacter (24)	*Aspergillus* (48)	*Candida*
Azotobacter (109)	*Cladochytrium* (385)	*Citeromyces*
Bacillus (263)	*Coprinus* (26)	*Cryptococcus*
Clostridium (216)	*Fusarium* (238)	*Kekkera*
Cytophaga (24)	*Helminthosporium* (230)	*Endomycopsis*
Edwardsiella (263)	*Neurospora* (162, 227)	*Hansenula*
Enterobacter (*Aerobacter* or *Klebsiella*) (16, 266, 369)	*Nowokowskiella* (385)	*Leucosporidium*
	Penicillium (385)	*Pachysolen*
	Phymatotrichium (385)	*Rhodosporidium*
Escherichia (155, 181)	*Phyctochytrium* (385)	*Rhodotorula*
Hafnia (263)	*Phytophthora* (385)	*Sporobolomyces*
Hyphomicrobium (24)	*Rhizophlyctis* (26)	*Sporidiobolus*
Micrococcus (255)	*Scopulariopsis* (184)	*Sterigmatomyces*
Nocardia (24)		*Trichospora*
Pasteurella (263)		
Providencia (263)		
Pseudomonas (260)		
Rhizobium (188)		
Sporocytophaga (24)		
Thiobacillus (354)		
Vibrio (262)		
Yersinia (263)		

[a] Very likely an incomplete listing.

[b] Based on Lodder's taxonomy (186). *Debaromyces* and *Pichia* species assimilate nitrite but not nitrate nitrogen.

[c] Evidence for assimilatory nitrate reduction by members of the genera identified in references 262 and 263 is based on their production of enzyme B.

denitrificans produces two distinguishably different nitrate reductases. When grown anaerobically at the expense of nitrate, the bacteria produce a particle-bound reductase which has the capacity to reduce chlorate as well as nitrate. With hydrogenase added, hydrogen serves as electron donor for experiments with cell-free extracts utilizing viologen dyes or flavine mononucleotide (FMN) as transport cofactors. Azide inhibition of the reduction of either anion by the particle-bound enzyme is competitive and reversible. A second, soluble nitrate reductase then demonstrated in extracts of aerobically grown cells is distinguished by its inability to reduce chlorate. Indeed, the presence of chlorate inhibits nitrate reduction by this soluble enzyme. The particle-bound reductase has a respiratory function in every bacterium that produces it and is designated enzyme A. The solubilized enzyme (designated B) has a nutritive rather than a respiratory role in a number of bacteria, although the oxygen sensitivity of the enzyme in a few species is thought to prevent unqualified acceptance of the nutritive role in every case (261).

Assay. A manometric method of differentiating enzymes A and B based on hydrogen-consuming, benzyl viologen-mediated reduction, or lack of reduction of chlorate is employed for assay of the activity in cell-free extracts that can reduce nitrate (269). By using this procedure, a number of *Aeromonas*, *Bacillus*, *Edwardsiella*, *Hafnia*, *Providencia*, and *Pseudomonas* species and *M. denitrificans* are found to produce enzyme B in minimal media containing nitrate as sole source of nitrogen. *Pasteurella* and *Yersinia* species produce enzyme B as well (263). By selecting from among these organisms the soluble enzyme produced by (i) the denitrifier *M. denitrificans*, (ii) the obligately aerobic, nonrespirer, but assimilator of nitrate, *P. putida*, and (iii) the facultatively fermentative *Hafnia* species, Pichinoty (260) confirms the inhibitory effects of chlorate, cyanide, and azide on enzyme B. He further notes that, whereas neither reduced nicotinamide adenine dinucleotide (NADH) nor reduced nicotinamide adenine dinucleotide phosphate (NADPH) serves as electron donor for assimilatory nitrate reduction, spectrophotometric assay based on changes in absorption at 340 nm is obviated. Even so, a colorimetric assay for nitrite production might logically be considered for assaying enzyme B. But, unlike enzyme A, which reduces nitrate along linear rates from the outset, the rates of nitrate reduction catalyzed by enzyme B diminish as a function of time after the initiation of reduction. Thus, a colorimetric method is deemed appropriate for the quantitative assay of enzyme A activity, but not for enzyme B (268). Two types of enzyme B are now discernible in several species of bacteria. Enzyme Bα is activated by 1 M NaCl, KCl, or CsCl, whereas Bβ is not (262).

Control. Regulation of the synthesis of enzyme B varies in different species (261). Production is constitutive in several species and repressible in others. In *M. denitrificans*, production is constitutive, and the quantity synthesized is unaffected by the presence or absence of ammonia or oxygen during growth. In *P. putida*, enzyme B is constitutively produced in aerated cultures, and growth in media containing complex nitrogenous compounds produces more of the enzyme than culture on minimal medium. In the facultatively fermentative *Aeromonas hydrophila*, *Edwardsiella tarda*, *Hafnia* species, *Providencia alcalifaciens*, and *Providencia stuartii*, enzyme B is also synthesized in low, but measurable, quantity during aerobic growth in complex medium. However, anaerobiosis and the presence of nitrate during growth greatly increase the output of enzyme B. Aeration suppresses activity of enzyme B in suspensions of these facultative bacteria that are able to reduce nitrate when incubated anaerobically, but the suppressive effect of oxygen is not inactivating. Cessation of aeration is followed by immediate restoration of activity at the same rate as the originally anaerobic system displays. Enzyme B thus is suggested to have anaerobic respiratory activity in these bacteria, but oxygen sensitivity seems a tenuous basis for such an hypothesis.

Another soluble, oxygen-sensitive nitrate reductase that transports electrons from reduced viologen dyes, but not NADH, is recovered from *R. japonicum* bacteroids from soybean nodules or from free-growing *R. japonicum* cells harvested from culture in minimal medium containing nitrate as sole nitrogen source. Cultures incubated at 0.2-atm oxygen pressure yield the maximal quantity of nitrate reductase. Protection of the reductase from oxygen by inclusion of reducing agents and metal chelators in the suspending media is necessary when assaying activity. To obtain active preparations, these protective agents must be present during rupture of the cells by repeated freeze-thaw cycles and simultaneous grinding with glass beads and during the subsequent clarifying centrifugation. Inclusion of Steapsin in the mixture increases the amount of enzyme recovered (188). Cyanide does not inhibit reduction of nitrate by this preparation. This may result from inactivation of the cyanide by the protective agents rather

than a lack of sensitivity (166), for cyanide does inhibit the activity of both respiratory and assimilatory nitrate reductases produced by other bacteria (63, 84, 138, 197, 260).

Nitrite and Hydroxylamine Reduction. One of the first properties ascribed to bacterial ferredoxin isolated from *Clostridium pasteurianum* was the capacity to participate, algae-like, in the reduction of nitrite and hydroxylamine to ammonia (216, 363). Hydrogen-hydrogenase supplies electrons, but this phenomenon has not been further investigated.

In *Escherichia coli*, nitrite reduction is carried out in a different manner that is more complex than might be anticipated. The bacteria can grow with nitrate, nitrite, hydroxylamine, or nitric oxide as sole nitrogen source (155, 181). These observations suggest that nitric oxide is an intermediate of assimilatory nitrite reduction and, furthermore, that it is reduced to hydroxylamine; but assays for this possibility have not been reported. *E. coli* can produce three different nitrite-reducing enzyme systems. A soluble, NADPH-specific fraction reduces nitrite, but appears from kinetic behavior to serve the cell primarily as a mechanism for sulfite reduction. Synthesis of this system is repressed by cysteine and sulfide (156). A reduced FMN ($FMNH_2$), or reduced viologen dye-linked, nitrite-reducing fraction has been discerned as well, but has not been further investigated. The third nitrite-reducing fraction is induced by anaerobic growth of *E. coli* in the presence of nitrate or nitrite and functions primarily in the NADH-dependent reduction of nitrite and hydroxylamine to ammonia (392). The slow rate at which exogenous hydroxylamine is reduced makes it doubtful that this compound is ever an exogenous substrate. Presumably, when produced by the reduction of nitrite, it does not leave the enzyme surface but is reduced to ammonia, the product released. The NADH-linked nitrite reductase is viewed as a nitrite toxicity-eliminating mechanism in *E. coli* (155) and only fortuitously as a contributor to nutrition.

Simultaneous with synthesis of the NADH-linked reductase, a superficially bound, low-potential cytochrome (c_{552}) is formed (108). When reduced, this cytochrome does not reduce nitrate but can be linked to the stoichiometric reduction of nitrite or hydroxylamine to ammonia (43), with concomitant release of carbon dioxide (88). Although the presence of nitrite during anaerobic growth increases the quantity synthesized by *E. coli* K-12 (233) (perhaps by increasing growth), the physiological necessity for participation of cytochrome c_{552} in nitrite reduction was questioned. Much of the pigment was separated from NADH-linked nitrite reductase which functioned then without it. When the cytochrome c_{552}-containing material was added back to reaction mixtures containing the enzyme from which it was separated, there was no stimulation of the rate of nitrite reduction (41). It was suggested that the NADH-nitrite reductase is a cytochrome *c* reductase with the added capacity to reduce nitrite. Recent studies with nitrite (*nirA*) and sulfite (*cysB*) reductase mutants indicate, however, that sulfite reductase is a gratuitous nitrite reducer that contributes little to nitrite reduction in growing cells (42). Moreover, the simultaneous loss in *nirA* mutants of the capacity for synthesis of both cytochrome c_{552} and nitrite reductase suggests that the pigment is involved in NADH-linked nitrite reduction in *E. coli* K-12. As would be expected, several *E. coli* strains do not produce cytochrome c_{552} when grown aerobically. In contrast, however, aerobically growing *E. coli* strain McElroy produces a somewhat similar but not identical cytochrome c_{552} that is capable of reducing nitrite (14). This pigment is presumably involved primarily in sulfite reduction, and even the McElroy strain loses the capacity to reduce either nitrite or sulfite aerobically unless stock cultures are maintained on sulfur-rich media.

Control of Synthesis of the Repressible Assimilatory Reductase in Enterobacter. Although a number of assimilatory nitrate-reducing species are recognized among the nondemanding heterotrophs, most of the studies of regulation of both synthesis and activity of the repressible assimilatory reductase have been conducted with *Enterobacter aerogenes* (also called *Aerobacter* or *Klebsiella* by various investigators [16, 266, 367, 369]). This bacterium has the dual capacity for assimilatory and respiratory nitrate reduction and can, in fact, carry out both activities simultaneously. Some years ago, a single reductase was postulated for this organism (265), but the observed differences in factors regulating synthesis of enzyme that carries out the two different activities are inconsistent with that notion. It has since been found (369) that when cells growing anaerobically in ammonia-containing minimal medium at the expense of nitrate respiration are suddenly aerated, nitrite production ceases immediately and growth lags for 3 h. The inhibition of reduction and lag in growth are explained by the further observation that both the synthesis and the specific activity of the respiratory reductase (assayed in cell-free extracts) are sensitive to oxygen. Sonication of the cells to

release the enzyme does not significantly damage the respiratory reductase. In contrast, the assimilatory reductase formed in cells grown aerobically with nitrate as sole source of nitrogen is relatively much less sensitive to oxygen, but quite sensitive to sonication and other manipulations. Aeration does not inactivate or inhibit the assimilatory reductase activity (determined in resting cells because of the fragility of the enzyme to sonication), although exposure to pure oxygen does.

The flow of electrons that reduce nitrate to ammonia proceeds by a different route than that followed by the electrons transported during respiratory reduction of nitrate. Although cytochrome b_1 is known to transfer electrons for respiratory reduction of nitrate, incubation of assimilatory nitrate reductase with electron donors and nitrate does not change the oxidation state of cytochrome b_1 (367).

An additionally significant difference in the regulatory mechanisms for assimilatory and dissimilatory nitrate reduction in *E. aerogenes* is indicated by the repression of synthesis of the assimilatory, but not the respiratory, reductase by ammonia. This would seem to argue for synthesis of different proteins for the assimilatory and dissimilatory functions. But, from data on pH and temperature optima for their activity, it was recently suggested that an identical protein may actually be synthesized in both instances but complexed differently by the cell to enable the protein to catalyze the two different functions (369). Isolation and comparison of purified subunit proteins from assimilatory and dissimilatory aggregates will be necessary for continued support of this hypothesis.

Synthesis of the assimilatory reductase has been found sensitive during growth to elevation of the temperature of incubation as well as the presence of ammonia. Growth of the pullulanase-producing strain of *E. aerogenes* at 37 C in nitrate minimal medium results in diminished synthesis of the assimilatory, but not the dissimilatory, reductase much below that seen in cells grown at 28 C (16).

Fungi

Distribution of Assimilatory Capacity Among Yeasts and Molds. As an aid to her taxonomy of the yeasts, Lodder considers the ability to assimilate nitrate nitrogen for growth as a primary determinative characteristic (186). She notes that one or more species in the genera listed in Table 1 are assimilatory nitrate reducers. Both sporogenous and asporogenous genera are represented. All these organisms assimilate nitrite as well as nitrate nitrogen; but, curiously enough, certain species of *Debaromyces* and *Pichia* assimilate nitrite but not nitrate nitrogen. Growth in a nitrate-containing medium does not induce the synthesis of nitrate reductase in species from these genera (306).

Determination of the distribution of the capacity for assimilatory nitrate reduction among filamentous fungi has not been reported so faithfully (Table 1). The original recipe for Czapek's medium, which was devised for the culture of *Aspergillus* and *Penicillium* species, called for sodium nitrate as the sole nitrogen source. Ammonium salts were later added to the formulation, however, to enable nonassimilators of nitrate to grow. Webster (385) indicates that, among the primitive fungi which are members of the Mastigomycotina, the chytrids such as *Phlyctochytrium*, *Cladochytrium*, and *Nowokowskiella* and members of the *Peronosporales* such as *Phytophthora* are nitrate assimilators. Fungi classified as *Saprolegniales*, *Leptomitales*, *Lagenidiales*, *Monoblepharidales*, and *Blastocladiales* are nonutilizers (26, 39, 184). Among the more complex fungi, ammonia is used preferentially over nitrate when ammonium nitrate is provided as nitrogen source in cultures of *Actinomucor repens*, *Alternaria tenuis*, *Aspergillus nidulans*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium solani*, *Neurospora crassa*, *Scopulariopsis brevicaulis*, and others (238, 240). Nitrate assimilation begins only when ammonia is depleted during the growth of several of these organisms, and addition of ammonia to a culture growing at the expense of nitrate stops nitrate (but not nitrite) reduction. This suggests catabolite repression of the first enzyme, nitrate reductase, but not the second enzyme in the assimilatory complex, nitrite reductase. In *Helminthosporium gramineum*, the response is different (230). Ammonia utilization proceeds much more rapidly than, but does not completely suppress, nitrate assimilation. A lesser amount of nitrate is reduced when ammonia is present, but the process does continue. A *Coprinus* species, *Fusarium niveum*, *Phymatotrichium omnivorum*, and *Rhizophlyctis rosea* utilize nitrite as a nitrogen source in culture as well as nitrate (26).

Regulation. Hansenula anomala. Among the nitrate-assimilating yeasts, only *H. anomala* has been analyzed for control mechanisms. Analogies as well as disparities are observed between the yeast and bacterial systems. Some years ago, Silver (306) found that growth of *H. anomala* at the expense of nitrate as sole source of nitrogen requires de novo synthesis of enzyme. Activity was assayed by simultaneous determination of rates of production of nitrite from nitrate and the concomitant

rates of spectrophotometric change brought about by oxidation of NADH (somewhat more effective than NADPH) in the presence of nitrate. Azide is an effective inhibitor. More recently (267) it was observed that, after aerobic growth of *H. anomala* in a minimal, mineral salts medium, with glucose and ethanol as carbon sources and nitrate as sole nitrogen source, cell extracts reduce nitrate at a significantly elevated rate at the expense of reduced benzyl viologen. Extracts of cells grown with ammonia as nitrogen source have minimal activity, and those obtained from yeast cells grown with urea as sole nitrogen source exhibit only a slightly increased rate of activity. This yeast produces urease, and the ammonia liberated by its action would be expected to repress synthesis of nitrate reductase. Extracts of the cells grown in media containing both nitrate and ammonia exhibit no more activity than extracts of cells grown with ammonia alone as nitrogen source. Similarly, extracts of cells grown in a medium containing urea and nitrate have no greater activity than those of cells grown with urea as sole nitrogen source.

Unlike the bacterial assimilatory reductase, which is soluble, the yeast nitrate reductase activity is associated with fragments of the mitochondrial fraction of the cells that are grown in nitrate minimal medium. Cytochrome *c* oxidase associated with the active fraction provides an added indication that the active material is mitochondrial in origin. Nitrate is assumed to act as inducer for the yeasts, but assays for derepression in resting cells incubated in the absence of nitrate or any other fixed nitrogen source are not reported. Slight activity is observed in extracts of cells grown with nitrite rather than nitrate serving as sole nitrogen source, but efforts to sort out the influence of ammonia and urea on this activity are inconclusive. Incubation of extracts containing the nitrate reductase produced by *H. anomala* in a medium containing reduced viologen dyes but lacking nitrate inactivates the enzyme. Neither nitrate reductase A nor B from bacteria is similarly sensitive to incubation with the reduced dyes in the absence of nitrate (257, 260).

Aspergillus nidulans. Apparently, wherever it is found in fungi, assimilatory nitrate reductase resides in a protein complex that exhibits several catalytic capabilities. The close association of nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.3) from *A. nidulans* with cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase, EC 1.6.2.3) activity, a phenomenom first noted in *Neurospora* by Kinsky and McElroy (162), is borne out during 300-fold purification of the enzyme as achieved by Cove and Coddington (48). After the first few steps of the purification procedure are passed, the ratio of cytochrome *c* reductase to nitrate reductase remains constant in the progressively more purified fractions. The elevated ratio observed during the early stages is explained by assuming that a certain quantity of cytochrome *c* reductase not associated with nitrate reductase is originally present in the extracts as well—but is soon eliminated. Phosphate is required for the activity of nitrate reductase from *A. nidulans* (191). The enzyme reduces chlorate as well as nitrate. This is remarkable mainly because the bacterial enzymes assumed to be assimilatory do not reduce chlorate and are, in fact, inhibited by the anion (260). Nitrite is a competitive inhibitor of nitrate reductase from *A. nidulans*.

The availability of ammonia controls a number of the pathways in the nitrogen metabolism of *A. nidulans*. Mutants derepressed for extracellular protease synthesis are derepressed for nitrate reductase production as well (40). But, more specifically, synthesis of the two ammonia-repressible enzymes required for reduction of (i) nitrate to nitrite and (ii) nitrite (through hydroxylamine) to ammonia has been examined in mutant as well as wild-type strains of *A. nidulans* (247). A regulator substance with a positive mode of action controls the synthesis of nitrate reductase in wild-type strains, and the control system is not located inside the nuclear membrane (47). Only two structural genes are known as sources of these activities, and either the regulator genes for both are missing or their gene products are incorrect in mutant *niiB* which has lost the capacity to reduce nitrate, nitrite or hydroxylamine. To approach the problem of control at the structural gene level, conidia of a nitrate reductase-inducible mutant (designated *niiA*$^-$) lacking the capacity to reduce nitrite or hydroxylamine were subjected to nitrosoguanidine to produce a nitrate reductase-constitutive strain, now designated *niiA*$^-$ *nir*^{c}. Studies of strains resulting from crosses of this mutant with wild-type as well as other mutants indicate the *nir*^{c} allele to be almost completely recessive in regulating synthesis of nitrite reductase apparently as a result of *nir* product being synthesized in sufficient quantity to control one structural gene, but not two (245, 247). A model that conforms to these findings presents *nir* as the regulator gene and assigns two functions to the gene product (46). As repressor, the presumably bifunctional product of *nir* thus prevents synthesis of nitrate and nitrite reductases by two structural genes. In the presence of nitrate, however, the product of *nir*, now assumed to act as inducer, permits

expression at the structural gene loci. Subsequently, it has been reported that the nitrate reductase protein molecule itself acts as corepressor in the absence of nitrate, but as coinducer when complexed with nitrate (49). The model is therefore adjusted to indicate that the product of *nir* is an inducer converted to a repressor by the nitrate reductase protein. Complexing of the protein with nitrate prevents conversion to repressor. The modified model does not explain induction of nitrate reductase by elevated concentrations of nitrate even in the presence of some ammonia, however, and a further provision is therefore included in the final model to stipulate that the product of the *nir* gene locus may be converted to repressor by both ammonia and nitrate reductase.

Although there has been some question as to whether Mo(V) or Mo(IV) is the active form (23), involvement of molybdenum in assimilatory nitrate reduction has been extensively investigated in fungi. Thus, the molybdate competitor, tungstate, is more toxic for *A. nidulans* growing at the expense of nitrate and hypoxanthine than any other sources of nitrogen. Provision of molybdate in the culture media reverses the tungstate toxicity. In attempting to explain this observation, Pateman and colleagues (246) found that mutation in any of five unlinked loci designated *cnx* can result in simultaneous loss of capacity for nitrate reduction and xanthine oxidation. One class of mutant, *cnxE*, which lacks the ability to grow on nitrate as nitrogen source, regains nitrate assimilatory function when cultures are supplied with molybdate. Although it functions well enough to support growth, the nitrate reductase isolated from extracts of mutant *cnxE* grown in the presence of molybdate displays diminished affinity for nitrate. Furthermore, a class of mutants termed *molB* is simultaneously both responsive to molybdate and partially defective in nitrate and hypoxanthine utilization (5). Hence, incorporation of molybdenum into nitrate reductase or a substance with controlling properties like those of nitrate reductase is postulated. Xanthine oxidase mutants do not respond to molybdate as do nitrate reductase mutants. This is considered by Holl (130) to be consistent with immunochemical evidence that nitrate reductase from *A. nidulans*, a dimeric molybdoflavoprotein (64), may not share a common subunit with xanthine oxidase in analogy with the nitrate reductase of *N. crassa* (161).

The question of whether the basic importance of molybdenum is structural or functional in nitrate reductase activity was examined by Downey (65), who finds that *cnx* mutants produce molybdenum-poor cytochrome *c*-reducing protein when induced with nitrate. But, in vivo complementation of the *cnx* mutant with a constitutive *nia* (nitrate reductase) mutant yields a normal nitrate reductase ("7.8*S* enzyme"). In contrast, in vitro complementation with extracts of these mutants yields a 7.8*S* enzyme that lacks nitrate reductase activity. These findings support a functional role in reduction for molybdate rather than its simply aiding in subunit assembly. (It would be interesting to determine if acid treatment of the *nia* product would provide a complementary component in analogy with the capacity described in the next section for in vitro complementation in *N. crassa* extracts [227].)

Extensive purification of nitrate reductase from *A. nidulans* (64) yields a flavoprotein (molecular weight, 197,000; Stokes radius, 6.4 mm; sedimentation value, 7.8*S* in a sucrose gradient) that reduces nitrate as well as cytochrome *c* derived from either *A. nidulans* or mammalian mitochondria. Unlike the NADH-linked, flavine adenine dinucleotide (FAD)-stimulated nitrate reductase in *H. anomala* (306), NADPH-linked reduction of nitrate is not associated with isolated mitochondria from *A. nidulans*.

The complexity of the regulatory mechanisms for nitrate reductase in *A. nidulans* has stimulated a number of ancillary questions. Considered together, the observations (i) that nitrate reductase is associated with the capacity for catalyzing other reactions and (ii) that none of the mutants at the structural gene locus yet isolated fail to synthesize a gene product lead, for example, to the suggestion that the gene coding for nitrate reductase may have a nitrate-independent but vital function in the growth of this fungus irrespective of the nitrogen source utilized. It follows logically from this suggestion, that it would be necessary that mutants unable to form the gene product be lethal. Testing this possibility in two ways, Downey and Cove (66) find no evidence favoring a need for the gene product required for nitrate reductase synthesis when the organism is grown in media containing ammonia or urea as nitrogen source. Neither do their results preclude the possibility entirely.

Neurospora crassa. NADPH-linked nitrate reductase. Studies with mutants reveal that, as in *A. nidulans*, the NADPH-linked nitrate reductase and cytochrome *c* reductase activities reside in one protein aggregate in this fungus. Five ultraviolet mutants have been isolated with loci designated *nit-1* to *nit-5* that control

synthesis of the components of the aggregate formed by *N. crassa* (316, 320). Acceptance of the existence of the aggregate is further supported by investigations (317) that indicate that nutritionally deficient mutants which require preformed amino acids and nucleotides for growth in minimal culture media also require them for synthesis of both these reductases. Moreover, inclusion of the amino acid analogue, 2-methylalanine, in the culture media represses synthesis of both nitrate reductase and cytochrome *c* reductase, but not the concurrent synthesis of an unrelated enzyme, isocitrate lyase. Furthermore, the specific activities of both these NADPH-dependent reductases remain nearly constantly related during their progressive heat inactivation in extracts. Nitrate-inducible cytochrome *c* reductase is separable by sucrose density gradient centrifugation from its constitutive counterpart enzyme in cell-free extracts (318). Comparing these two cytochrome *c*-reducing enzymes, it may be seen that the activity of the nitrate-induced, but not the constitutive enzyme, is stimulated by FAD; and unlike the constitutive enzyme, of course, the induced cytochrome *c* reductase is tightly associated in fractionated extracts with nitrate reductase. Investigation of fractions derived from extracts of several nitrate reductase mutants suggests that the NADPH-linked nitrate reductase itself comprises an aggregate of two proteins. Synthesis of one that exhibits benzyl viologen-linked nitrate reduction is controlled by the *nit-1* locus. Production of the other, which displays cytochrome *c* reductase activity as well, is regulated by the *nit-3* locus. A respiratory function is ascribed to this benzyl viologen-dependent nitrate reductase, which contains iron as well as molybdenum (231). Anaerobic respiration is not considered a property of fungi. The report of a probable respiratory role for succinate-dependent nitrate reductase in the basidiomycete, *Hygrophorus conicus*, represents the only other suggestion of such a role for nitrate reduction encountered in the preparation of this review (207).

It is reasonable to suppose that because the key nitrogenous intermediate in amino acid, nucleotide, and coenzyme synthesis (ammonia) represses utilization of nitrate as a nitrogen source, the organic nitrogenous nutrients resulting from ammonia assimilation should serve as repressors in nutritionally wild-type *N. crassa* as well. And, consistently, various amino acids do repress synthesis of assimilatory nitrate reductase as effectively, or nearly so, as ammonia (338). As would further be expected, production of nitrate-inducible cytochrome *c* reductase is concomitantly repressed by the amino acids. Synthesis of catalase (which occurs subsequent to synthesis of nitrate reductase and probably in response to the consequent production of hydrogen peroxide) is also repressed by ammonia and amino acids. But unlike the synthesis, the activity of nitrate reductase in cell-free extracts is not inhibited by assimilatory products, i.e., ammonia, amino acids, or nucleotides (339). The intermediates of assimilatory nitrate utilization, nitrite and hydroxylamine, are effective competitive inhibitors, with the latter more effective than nitrite.

Once synthesized, assimilatory nitrate reductase is not stable in culture if either nitrate is depleted or ammonia is provided. Incubation of wild-type mycelia in culture media containing either ammonia or no fixed nitrogen source results in rapid inactivation of existing nitrate-induced, NADPH-linked nitrate reductase and its associated activities, NADPH-cytochrome *c* reductase and reduced benzyl viologen-dependent nitrate reductase (341). Inclusion of the protein synthesis inhibitor cycloheximide slows the rate of inactivation which is directly related to ammonia concentration. In a mixture of ammonia and nitrate, the rate of inactivation is unrelated to the nitrate concentration. In mutants unable to assimilate nitrate nitrogen, the two associated activities (cytochrome *c* reduction and reduced benzyl viologen-linked reduction of nitrate) are not inactivated by the addition of ammonia or withdrawal of nitrate. In these strains, only the activity of the portion of the protein aggregate that effects NADPH-linked nitrate reduction is thus sensitive to influence by nitrogen source.

The repressive and otherwise controlling influence of ammonia on nitrate assimilation is not simple, and there is no single, specific point of sensitivity to its effects. Reasoning that the regulatory influences of nitrate and ammonia might be exerted upon transcription, translation, or stability of the extant enzyme, Subramanian and Sorger (342) took into account the different sites of action of the two antibiotics, actinomycin D, which prevents transcription, and cyclohexamide, which inhibits translation of message, to attempt to design differentiating experiments. They find, however, that although nitrate reductase is synthesized at a low rate in the absence of a nitrogen source, it is produced optimally only when nitrate is supplied as nitrogen source during both transcription and translation. Ammonia inhibits the increased capacity for synthesis of nitrate reductase that results from the presence of nitrate. Even after the increased capacity for synthesis becomes

operational, its functioning as well as the activity of enzyme once it is synthesized both require nitrate for stability. In addition, both the increased capacity for synthesis and the activity of the reductase are made unstable by the presence of ammonia. Only a low level of nitrate is required to enhance capacity for synthesis, but a high level is required to maintain functional stability of enzyme once it is formed.

Experiments designed to reveal the point at which molybdate influences the synthesis of nitrate reductase indicate that the complex protein is produced even in the absence of molybdate or the presence of a toxic quantity of tungstate, but the folding of the polypeptide chains is different from that obtained when the molybdate supply during growth is adequate and available (340). The complex molybdenum-deficient protein thus obtained displays NADPH-dependent cytochrome *c* reductase activity (which is not molybdenum related), but neither NADPH- nor benzyl viologen-linked nitrate reductase activity is demonstrated. Addition of molybdate to suspensions of the protein in vitro fails to restore nitrate reductase activity. Molybdenum seems thus to have a structural as well as functional role. The functional role is further supported by experiments with enzymes derived from the large-scale purification of NADPH-linked nitrate reductase which yielded active material containing a *b*-type cytochrome similar to yeast and mammalian *b*-type pigments (90). When reduced, the cytochrome has an α-band absorption peak at 557 nm, a β peak at 528 nm, and a Soret peak at 423 nm. Participation of this b_{557} cytochrome in the assimilatory electron flow is postulated to occur as follows: NADPH $\rightarrow$ FAD $\rightarrow$ Cytochrome $b_{557} \rightarrow$ Mo $\rightarrow NO_3^-$.

The complexity of the NADPH-nitrate reductase protein and the ability of mutants to synthesize subunits enabled Ketchum et al. (161) to assay for assembly of *Neurospora* protein in vitro with other subunit enzyme systems derived from various sources, including animals. Starting material is subunit protein that displays a cytochrome *c* reductase, but not nitrate reductase activity, and is produced by nitrate induction in mutant *nit-1*. Addition of a second component produced by other mutants or wild-type *Neurospora* (and found to be dissociable from its native state by acid treatment) completes the assembly and provides a complex protein capable of catalyzing nitrate reduction. The latter subunit is replaceable by other molybdenum-containing enzymes such as bovine milk and rabbit and chicken liver xanthine oxidizing and aldehyde oxidase systems that have been partially purified and treated with 1 N HCl. The assembly, comprising nitrate-induced *nit-1* subunit and a complementary animal component, yields a 7.8*S* protein containing cytochrome b_{557} and displaying NADPH-, $FADH_2$-, or reduced methyl viologen-dependent nitrate reductase activity. It has now been determined (227) that a remarkable variety of acid-treated, molybdenum-containing enzymes such as bacterial nitrogenase, bovine liver sulfite oxidase, *E. coli* respiratory nitrate reductase, and green plant nitrate reductase also complement *nit-1* protein to yield a 7.8*S* protein capable of NADPH-dependent nitrate reduction. A number of amino acid-molybdenum complexes and molybdenum-free enzymes tested as controls do not replace the molybdenum-containing systems. These observations may reflect the limited number of structural configurations possible for the physiologically effective binding of molybdenum for an electron-transporting role.

Nitrite reductase. Progress in the study of assimilatory nitrite reduction has been slow because, for many years, methods for the assay of nitrite reductase were not satisfactory. Cook and Sorger (45) thus devoted time to evaluation of the influence on reproducibility of the colorimetric method for nitrite determination exerted by the use of dithionite as electron donor. Dithionite was added to reaction mixtures containing benzyl viologen, buffer, enzyme, and nitrite. This procedure was found acceptable for their purposes and was employed in studies designed to determine if nitrite reductase is induced by nitrate or derepressed by a period of nitrogen starvation. Their data favor derepression by nitrogen starvation. A nitrogenous inducer is reportedly not required to initiate synthesis by wild-type *N. crassa* (strains 74A and 3.1a), and the availability of Casamino Acids during incubation apparently does not completely repress synthesis of nitrite reductase. Once produced, the nitrite reductase is as active in the presence of nitrate, ammonia, or Casamino Acids as in their absence. In contrast, Garrett (89) presents the diametrically opposed view that mycelia of wild-type *N. crassa* (strain 5297a) incubated in the absence of a nitrogen source do not synthesize nitrite reductase. In the presence of nitrate or nitrite, however, the mycelia are induced to form nitrite reductase that is demonstrable by either NADPH- or reduced benzyl viologen-dependent reduction of nitrite. Ammonia represses synthesis. NADPH-hydroxylamine reductase activity is similarly induced and repressed, and the protein that catalyzes hydroxylamine reduction is consid-

ered to be identical with NADPH-nitrite reductase. Similarities between production and activity of the nitrate and nitrite reductases of *Neurospora* and *Aspergillus* are said to be close.

A number of mutants recently isolated (195) accumulate but cannot reduce nitrite. One particularly interesting isolate accumulates hydroxylamine. Because a single protein is assumed to reduce nitrite, first to hydroxylamine and then to ammonia, study of the nitrite reductase from this mutant may provide useful information on (i) the mechanism of the first reductive step in nitrite assimilation as well as (ii) the nature of the defect that prevents the mutant's enzyme from proceeding with the second step, reduction of hydroxylamine to ammonia.

Photosynthetic Microorganisms

Bacteria and Blue-Green Algae. Nitrate reduction by photosynthetic bacteria has been investigated but little. The single available report indicates that extracts of *Rhodospirillum rubrum* grown either aerobically or anaerobically in light with nitrate as the sole source of nitrogen contain nitrate reductase activity associated with the chromatophores. The enzyme utilized NADH or reduced viologen dye as electron donor (152). Flavines, a *b*-type cytochrome, and nonheme iron appear to be involved. It is suggested that a single enzyme serves for either assimilatory or dissimilatory reduction, although this has not been true for a number of other types of bacteria.

Assimilation of nitrate nitrogen by prokaryotic algae has been studied only a bit more extensively. In extracts of *Anabaena cylindrica* grown on nitrate-containing medium in the absence of ammonia, the complex, green plant-like nitrate reductase (NADH:nitrate oxidoreductase, EC 1.6.6.1) remains particulate (126, 234). Treatment with Triton X-100 removes the diaphorase from the particulate complex, but the remaining fraction retains the capacity for reduction of nitrate at the expense of reduced viologen dye (125). *Microcystis aeruginosa* also produces NADH-linked nitrate reductase (274). In *A. cylindrica*, the rates of nitrate reduction and assimilation vary directly with photosynthetic activity (125), and synthesis of ferredoxin-linked nitrite reductase is induced by the simultaneous presence of nitrite and absence of ammonia (234).

Genetic studies of factors controlling blue-green algal nitrate reduction have not been numerous but seem promising for the future. Mutants of *A. nidulans* (128) and *Agmenellum quadruplicatum* (136, 327) lacking nitrate and nitrite reductases have been isolated from populations treated with nitrosoguanidine or ultraviolet light.

Other Microscopic Algae. Pyridine nucleotide-linked nitrate reductase. Although both are much inferior to ammonia, nitrate and nitrite will serve (each as well as the other) as a nitrogen source for the growth of the microscopic eukaryotic as well as other algae (25). Assimilation rates in these organisms are also directly related to light intensity. In *Dunaliella tertiolecta*, the rate of light-stimulated nitrate reduction is 20-fold greater than that occurring in the dark. In addition to the response to light, concomitant fixation of carbon dioxide is necessary for nitrate reduction to proceed in this alga (104, 241) and in *Ankistrodesmus braunii* (215), *Chlamydomonas reinhardi* (357), *Chlorella fusca* (formerly *C. pyrenoidosa*) and other species (105, 107, 337), *Skeletonema costatum*, and *Coccolithus huxleyi* (79). The dependence of the rate of nitrate reduction on photosynthesis in *Ditylum brightwellii* (77) and *D. tertiolecta* (241) is revealed by the direct relation between (i) nitrate and nitrite reduction and (ii) growth rate and ribulosediphosphate carboxylase activity. Partial inhibition of nitrate assimilation by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) is thus an indirect consequence of inhibition of photosynthesis by DCMU (215). It is the carbon skeletons made available for assimilation of the ammonia resulting from nitrate reduction that appear as the influential factors resulting from carbon dioxide fixation (or from adding acetate or mobilizing carbon reserves in cells that are reducing nitrate in the dark) (356, 357). Uncouplers of phosphorylation that do not influence cell-free reductase activity but diminish rates of nitrate and nitrite assimilation also appear to function indirectly. By decreasing rates of synthesis of all the algal carbon compounds, the uncouplers lower the supply of carbon skeletons that may be expected to take up the ammonia generated by nitrate reduction (3, 158). Photosynthesis in *C. fusca* is most sensitive to inhibition by carbon monoxide in the presence of nitrate (374).

Until it is incorporated into organic compounds, the presence of ammonia from any source represses nitrate and nitrite uptake (129), inhibits synthesis, and initiates inactivation of nitrate reductase in algal cells (187). The enzyme is rapidly reactivated when ammonia is depleted. The presence of nitrite also represses nitrate uptake in several algae (76) and the availability of urea represses assimilation in *Chlorella vulgaris* (311). Only when ammonia is absent does *D. brightwellii* consume nitrate

(which can be concentrated prior to reduction in the vacuole as well as the cytoplasm) and nitrite (which neither enters the vacuole nor concentrates in the cytoplasm). Nitrate inhibits nitrite reduction in cell-free extracts, but growing cells can utilize both simultaneously (77).

The relative effectiveness of the uptake mechanisms for nitrate revealed by calculation of half-saturation constants can be correlated with successions and with the aquatic areas from which clones of *D. brightwellii* (75, 76), *Cyclotella nana*, *Fragilaria pinnata*, *Bellerochia* species and others (31, 78, 80) are isolated. Both intraspecific clones and unrelated species from offshore marine environments where nitrate concentrations are low take up nitrate more rapidly than their counterparts isolated from tidal, estuarine, or freshwater sources where nitrate concentrations are higher.

The ability to assimilate ammonia is more stable to lowered temperature of incubation in the thermal alga *Cyanidium caldarium* than the capacity for assimilating nitrate (289). Ammonia will serve as nitrogen source for growth at 24 C, whereas nitrate serves only at 30 C or above (optimal growth temperature is 45 C).

In the absence of ammonia, the presence of nitrate induces the synthesis of the green plant-like nitrate reductase in *Biddulphia aurita* (190), *C. fusca* (360, 396), *C. reinhardi* (129), *C. vulgaris* (311), and *D. brightwellii* (75). Production is minimal in dark-growing cells (148). An exogenous supply of nitrate is not required for initiating synthesis of nitrate reductase in nitrogen-depleted *C. fusca*, *C. vulgaris*, and *A. braunii*. Two explanations are advanced: (i) the lack of ammonia derepresses synthesis or (ii) internal nitrate is said to result from the oxidation of nitrogenous reserves. The internal supply is thought then to induce nitrate reductase production (159, 232). Two nitrate reductase systems are postulated (one inside and one outside the chloroplasts) in *D. tertiolecta* (105), although *C. fusca* produces enzyme that appears identical whether it grows in the light or in darkness at the expense of glucose (112).

Several of the algae are apparently not restricted to use of NADH-dependent nitrate reductase. In addition to NADH, NADPH and reduced ferredoxin are reportedly accepted as electron donors for the reduction of nitrate by enzymes from certain algae (288). The possibility that NAD is the significant intermediate carrier is not rigorously excluded, but the molybdoflavoproteins isolated from *D. tertiolecta* and *A. braunii* retain the capacity to accept electrons from both NADH and NADPH for nitrate reduction even at the highest degree of purification obtained (182). Unlike other preparations, the nitrate reductase from *D. tertiolecta* (molecular weight estimated at 500,000) does not transfer electrons from reduced viologen dyes.

A complex NADH-linked nitrate reductase (molecular weight also estimated at 500,000) with initially poor activity is obtained from extracts of *C. fusca* (394), and a similarly complex NADPH-dependent reductase is produced by *C. caldarium* (290). The *C. fusca* enzyme is activated by incubation at pH 6.2 to 6.5 in the presence of nitrate, or phosphate (214, 375, 376), or ferricyanide (144), which also activates the nitrate reductase from *C. reinhardi* (129). The enzyme from *C. caldarium* is activated by incubation with phosphate and urea (290). *C. vulgaris* produces an NADH-dependent nitrate reductase in the low-activity form. The enzyme is slowly activated up to 50-fold by phosphate, nitrate, Mg^{2+}, and adenosine diphosphate (ADP). Addition of flavines is not required for maximum activity (313).

In analogy with the enzymes from fungi, the somewhat purified nitrate reductase from several algae has associated cytochrome *c* reductase activity (nine times more active in *C. fusca* than in *C. vulgaris*), utilizes FAD as intermediate carrier and is stabilized against heat inactivation by the presence of FAD (395), contains molybdenum (27) and cytochrome b_{557}, and reduces chlorate, bromate, and iodate (315). Nitrate competitively inhibits chlorate reduction. The flavine-containing portion of the complex has diaphorase activity that can be separated from the molybdenum-containing reductase which retains the capacity to accept electrons from reduced viologen dyes for the reduction of nitrate. Heating in the range between 42 and 64 C inactivates the flavine-linked oxidation of NADPH carried out by enzyme from *C. caldarium* (288, 291). Capacity for reduced viologen dye-linked reduction of nitrate is retained by both these fractions, but exposure to 65 to 75 C inactivates the capacity even for reduced dye-dependent reduction by the *C. caldarium* fraction.

The indispensable nature of molybdenum as a component of algal nitrate reductase is revealed in a number of ways. The concentration of the enzyme varies with the quantity of molybdate provided (27). When ^{99}Mo (as molybdate) is added to ammonia-depleted cultures of *C. fusca*, it is incorporated and appears only in nitrate reductase (4). In the absence of molybdate, inactive NADH-nitrate reductase is formed (370). If the molybdate antagonist, ^{185}W-labeled tungstate, is provided during in-

duction of the synthesis of nitrate reductase, the label is incorporated into an inactive nitrate reductase that retains nonmolybdenum-requiring diaphorase activity (242). Mild heat treatment of nitrate reductase from *C. fusca* yields a preparation that can then incorporate hydrosulfite-reduced molybdenum into its structure and make use of the electrons from the reduced metal to reduce nitrate (4). Finally, as might be expected from the demonstration of iron as well as molybdenum in the nitrate reductase from the bacterium *M. denitrificans* (85) ^{59}Fe (provided as ferrous ion) is also incorporated into nitrate reductase by *A. braunii* (397).

After stimulation to full activity by oxidizing agents, the activity of nitrate reductase in cell-free extracts of *C. fusca* is competitively inhibited by a variety of anions (azide, cyanate, thiocyanate, and nitrite) as well as chlorate and oxidized cytochrome *c*. KCN inhibits activity, and both KCN and hydroxylamine slowly inactivate the enzyme. Inactivation is then slowly reversed by incubation with nitrate. In high concentrations, pyridoxal phosphate inhibits both the nitrate and cytochrome *c*-reducing capacity of the complex enzyme (314).

Ferredoxin-dependent nitrite reductase. Uptake and reduction of nitrite are also directly related in algae to the rates of photosynthesis and carbon dioxide fixation. Decreasing the rate of carbon dioxide assimilation by either restricting the quantity of the gas provided or inhibiting with iodoacetamide the rate of reduction of carbon dioxide concurrently decreases the rate of reduction by whole cells of *C. fusca* (336). The rate of induction of nitrite reductase is directly linked to the stepwise deoxyribonucleic acid (DNA) synthesis in the light in synchronously dividing *C. fusca* cells. Exposure to actinomycin D reveals a close relationship between the rates of inducibility of ribonucleic acid (RNA) and nitrite reductase syntheses. This is interpreted as indicating that the gene for nitrite reductase is accessible for transcription, and the messenger RNA coded for nitrite reductase is produced only when DNA is replicating (169). Production of the enzyme is minimal in cells growing synchronously in the dark. For reasons not yet apparent, nitrite reductase activity is stimulated independently of any photosynthetic effect by short wavelength (465 nm) light (335, 337).

Molybdenum is not a component of nitrite reductase and, as anticipated, the presence of tungstate does not affect nitrite nitrogen incorporation (27, 370). But, the supply of iron in the culture medium influences the synthesis of nitrite reductase in *C. fusca* more than the formation of nitrate reductase (28, 157). As would be expected, ^{59}Fe (supplied as ferrous ion) is incorporated into nitrite reductase if supplied during induced synthesis (394).

Growth dependent upon either nitrate or nitrite initiates synthesis of ferredoxin-linked nitrite reductase in *C. fusca* (169) and *B. aurita* (190), but it is nitrite specifically that induces synthesis. Hydrogenase functions with the enzyme from glucose-grown *C. fusca* only in the presence of carbon dioxide (329). Neither NADH nor NADPH serves as electron donor, although reduced viologen dyes can be substituted for ferredoxin (394). Nitrite reductase appears to be much less complex than nitrate reductase. A molecular weight of 63,000 has been estimated for the enzyme from *C. fusca* (394), whereas that from *D. teriolecta* is approximately 70,000 (106).

DISSIMILATORY REDUCTION BY BACTERIA

Distribution Among Various Species

Respiratory Reduction to Nitrite. The mean energy yield for the transfer of a molar equivalent of electron from an organic compound to oxygen is 26.5 kcal (250). Calculations based on data presented by McCarty (203) further indicate that the mean yield for the transfer of an equivalent of electron from an organic compound (i) to nitrate is approximately 18 kcal, (ii) to sulfate, 3.4 kcal, and (iii) to carbon dioxide, 2.4 kcal. Considering that these are the major inorganic oxidants supporting respiration in bacteria, it is thus consistent that the number and variety of the nitrate reducers should be greater and more diverse than the sulfate and carbon dioxide reducers. It is true as well that a greater number of bacterial species capable of dissimilatory than assimilatory nitrate reduction exist, but explanations for this are not so obvious. Survey of the taxonomic descriptions in Bergey's Manual (24) for notation of the ability of bacteria to produce "nitrites from nitrate" reveals the property in certain species in the genera listed in Table 2. A few of these may actually be denitrifiers in which further reduction has not been recognized. There appear to be no correlations upon which taxa may be established between capacity for nitrate respiration and the specific pathway of attack on carbohydrates utilized by various bacteria (171). Actually, the value of determining nitrate respiration as a general taxonomic criterion does not appear great, although it may be useful in restricted instances. Chamroux (33) notes that capacity for nitrate

TABLE 2. *Genera of chemosynthetic bacteria containing species reported to reduce nitrate dissimilatively and to denitrify*

Nitrate respiring ($NO_3^- \rightarrow NO_2^-$)		Denitrifying ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$)
Achromobacter (24)	*Haemophilus* (308)	*Achromobacter* (140)
Actinobacillus (24)	*Halobacterium* (24)	*Alcaligenes*[a] (200)
Aeromonas (24)	*Leptothrix* (24)	*Bacillus* (69, 377)
Agarbacterium (24)	*Micrococcus* (24)	*Chromobacterium* (24)
Agrobacterium (24)	*Micromonospora* (24)	*Corynebacterium*[b] (285)
Alginomonas (24)	*Mycobacterium* (92, 175, 280)	*Halobacterium* (24)
Arizona (263)	*Nocardia* (24)	*Hyphomicrobium* (322)
Arthrobacter (24)	*Pasteurella* (24)	*Micrococcus* (255, 262)
Bacillus (239, 302)	*Propionibacterium* (24)	*Moraxella* (131)
Beneckea (24)	*Proteus* (56)	*Nitrosomonas*[b] (133, 390) (Not known if observed nitrite reduction to nitric oxide and nitrous oxide serves respiratory function)
Brevibacterium (24)	*Providencia* (263)	*Propionibacterium* (24)
Cellulomonas (24)	*Pseudomonas* (24)	*Pseudomonas*[b] (82, 110, 268)
Chromobacterium (24)	*Rettgerella* (83)	*Spirillum* (379)
Citrobacter (263)	*Rhizobium* (53)	*Thiobacillus* (1, 137)
Corynebacterium (153)	*Salmonella* (332)	*Xanthomonas* (379)
Cytophaga (24)	*Sarcina* (24)	
Enterobacter (*Aerobacter* or *Klebsiella*) (265, 334, 369)	*Selenomonas* (24)	
	Serratia (239)	
	Shigella (263)	
Erwinia (24)	*Spirillum* (38)	
Escherichia (305)	*Staphylococcus* (174, 391)	
Eubacterium (24)	*Streptomyces* (176)	
Flavobacterium (24)	*Vibrio* (24)	
	Xanthomonas (24)	

[a] One species reduces nitrite to nitrogen but does not reduce nitrate (34).
[b] Certain species and strains yield nitrous oxide as a terminal product of reduction.

respiration does not set marine bacteria apart as a distinct group. She finds representative nitrate reducers spread through all of the five groups of marine isolates established in her studies by their requirements for and tolerance of the inorganic ions in sea water. Possession of the property was assayed in recent, long-term, collaborative efforts to bring order to the taxonomic disarray of the *Actinomycetales* (304), but disagreement on interpretation of results by a number of the collaborators resulted in its rejection as a differential criterion. Within that group of workers, those who employ numerical taxonomy (176) do find some value in assays for nitrate reduction in the differentiation of species in the genus *Streptomyces*, however.

Assays for nitrate respiration are most effectively used in clinical laboratory microbiology, particularly for differentiating *Mycobacterium tuberculosis* from closely related species (24). *Corynebacterium* species may also be differentiated in some degree by production of nitrate reductase (153). Technical improvements have recently been introduced into the assay procedures employed. For example, addition of short-chained fatty and related organic acids to nitrate-containing culture media increases the nitrate reductase activity of *M. tuberculosis* and unexpectedly reveals that one of several other species thought to lack the ability can reduce nitrate when provided with the appropriate electron donor. Thus, *Mycobacterium scrofulaceum* is found to reduce nitrate in cultures containing lactic acid as electron donor, but not in media without it (20). Although nitrate reduction is determined conventionally in broth tube assays, use of paper strips impregnated with nitrite-detecting reagent is a recent innovation for screening mycobacterial isolates for classification. Their stability, simplicity of use, and rapidity of response recommend the test strips for wider employment (280).

Association of nitrate reductase with other physiological phenomena has been noted in mycobacteria. Lysogeny in several strains of *Mycobacterium smegmatis* is accompanied by a decrease in the capacity for amidase, nitrate reductase, and arylsulfatase production (92, 146). In addition, only the smooth-colony producers among both *Mycobacterium fortuitum* strains produce nitrate reductase, whereas both smooth and rough colony producers in the group III nonphotochromogenic isolates produce the enzyme (175). Loss of the capacity for nitrate reduction by the rough-colony producing *M. fortuitum* strains is correlated with, and may

result from, lysogeny. The practical value of this observation lies, however, in the indication that colonies should be isolated from populations with low or variable nitrate reductase activity and assayed individually as an aid to diagnosis of the identity of *M. fortuitum* isolates.

According to Bönicke and Kazda (21), assays for nitrite reduction in media containing a variety of sugars are also valuable. The rapidly growing *Mycobacterium* species differ in their ability to utilize 17 representative carbohydrates, which suggests the identification of species of this group may be aided by use of this carbohydrate-nitrite reductase test.

Among several enzymes produced by wild-type, oxytetracycline-sensitive *Staphylococcus aureus* isolates, nitrate reductase and lactate dehydrogenase isoenzymes, whose activity is resistant to inhibition by oxytetracycline, are the ones that arise and persist in emergent oxytetracycline-resistant strains. In contrast, malate dehydrogenase and tellurite reductase are not discerned in the resistant strains (174). Because all four are presumably membrane-associated enzymes, location in the cells does not appear to be a governing factor in this association of isoenzyme production and development of resistance. In work with bacteria other than mycobacteria, tests with paper strips impregnated with nitrite-detection reagent also compare quite favorably for precision with the conventional tube assays. Greater rapidity of use thus recommends the strip tests for presumptively distinguishing between the reductive *S. aureus* and nonreductive *Gaffkya* species and between the reductive members of the *Enterobacteriaceae* and nonreductive gram-negative isolates such as *Herella* and *Mima* species (391).

The hypothesis that nitrate reductase may reduce ferric ion has recently been advanced. Consistent with this notion is the direct correlation between the upsurge in ability to reduce ferric ion and the onset of chlorate-induced production of respiratory nitrate reductase in *E. aerogenes*, *E. coli*, *Serratia marcescens*, *Bacillus cereus*, *Bacillus polymyxa*, and *Pseudomonas aeruginosa*. Moreover, *Bacillus pumilis* and *Bacillus sphaericus*, which do not reduce nitrate, reduce very little iron and are not stimulated to reduce more by prior incubation with nitrate (239). The simultaneous presence of nitrate and iron decreases the quantity of iron reduced (possibly by competition) during growth of the chlorate-inducible bacteria. Nitrate reductase-less mutants derived from the active species retain only a function of the parent strains' slight but constitutive capacity for reduction of ferric iron. The presence of nitrate does not diminish the capacity of the mutants to reduce that small quantity of ferric ion, using what is considered to be a second, but yet unspecified, mechanism.

The compound which joins with sulfanilic acid to comprise the active components of the nitrite assay reagent, α-naphthylamine, is carcinogenic. This prompted Parrakova, Mayer, and Janouskova (243) to test a noncarcinogenic compound, 1-naphthylamine 7-sulfonic acid or Cleves acid, as a substitute, as suggested by Crosby (52). The substitute reagent is reportedly as effective as, or superior to, α-naphthylamine for the assay of nitrate reductase in several species in *Enterobacteriaceae*.

Denitrification. Bacterial isolates reported to be members of the genera listed in Table 2 are known to release nitrogen (or nitrous oxide) by respiratory nitrate reduction (81, 131, 225, 323, 343, 354, 393). The property fits poorly with other physiological aspects of certain of these (e.g., the bacilli), and Verhoeven (377) thus suggests a new genus, *Denitrobacillus*, to accommodate the species he considers unique, *Bacillus licheniformis*.

Over several decades, a few denitrifying *Pseudomonas* species were isolated in routine surveys of soils, freshwater, salt marsh mud, and seawater, but recently a different type of searching prompted by practical considerations has been conducted. Denitrifiers thought to have a significant metabolic influence in various aquatic environments have thus been obtained. From activated sludge, for example, von Mechsner and Wuhrmann (379) obtained denitrifying bacteria believed to be *Spirillum*, *Pseudomonas*, and *Xanthomonas* species and noted variance among them in their response to oxygen. Some require anoxia for the initiation and continuation of denitrification; others are more tolerant of limited quantities of oxygen. One spiraled bacterium continues denitrifying at an oxygen tension as high as 153 mm. As might be anticipated (36, 54, 98, 204, 253, 254, 275), nitrite accumulates transiently early in the denitrifying period of several of the isolates.

Interest in eliminating nitrate from sewage prompted a search for the specific bacteria that release nitrogen at the expense of methanol—the simplest, most easily manageable organic source of electrons that can be used for this purpose (its metabolic products are cells, carbon dioxide, and water). Surprisingly, anaerobic enrichment cultures supplied with methanol and nitrate yield pure cultures of a filamentous, budding *Hyphomicrobium* species that stoichi-

ometrically releases nitrogen from nitrate or nitrite and carbon dioxide from methanol (322). A recently isolated *Pseudomonas* species has the capacity to utilize benzoate and several other ring compounds as electron donors for anaerobic denitrifying growth (352, 353). This is unexpected because oxygenation is presumably required for metabolic cleavage of the ring structure. Finally, an unidentified gram-negative bacterium quite like *Moraxella kingii* is isolated on occasion from pharyngeal and urinary sources on media selective for *Neisseria*. The capacity of this isolate for denitrification sharply distinguishes it from other related bacteria (131).

Nitrate Reductase

Oxygen Availability and Other Physiological Influences on Synthesis and Activity. Despite general acceptance of dissimilatory nitrate reduction as an anaerobic alternative to oxygen-linked respiration, there was for many years no general agreement on the physiological locus of sensitivity to oxygen nor the degree of sensitivity at the locus. In 1955, a simple but telling experiment by Collins (44) revealed that, even in presumably aerated cultures, the ability of *P. aeruginosa* to gain the capacity for nitrate respiration was directly related to the shape of the culture flask employed. Growth in shaken flasks with gradually more confining structures yielded cells that approached the control (cells respiring nitrate anaerobically) in nitrate-reducing ability. Nitrate reduction is then directly proportional to the ability of the flasks for limiting aeration of their contents when shaken.

E. aerogenes. It is now known that oxygen inhibits onset of the synthesis in *E. aerogenes* of the nitrate respiratory system. In anaerobic cultures containing nitrate, chloramphenicol and fluorophenylalanine also inhibit synthesis of nitrate reductase. Once formed, nitrate reductase in whole cells is not inactivated by aeration or exposure to chloramphenicol, however (256, 265, 266). The suppressive influence of oxygen on biosynthesis of enzymes that contribute to this type of respiration is, of course, not surprising. It is, in fact, generalized and not limited to affecting nitrate respiration. In *E. aerogenes* and a number of other bacteria (57, 58, 86, 150) oxygen represses the biosynthesis of several other alternative systems as well (e.g., reductases for nitrite, nitrous oxide, tetrathionate, thiosulfate, fumarate and acetoin, and hydrogenase activity).

E. coli. In strain K-12, biosynthesis of nitrate reductase is derepressed in the absence of nitrate by anoxia alone; but in the presence of nitrate, synthesis is initiated when oxygen tension is significantly lowered even before anoxia is reached (305). In mutants selected for electron-transport lesions, nitrate reductase is produced in the presence of oxygen (307). It is suggested, therefore, that oxygen functioning as electron acceptor is the repressing agent rather than oxygen per se. Under anaerobiosis, the presence of nitrate induces a greatly increased production of nitrate reductase in wild types. When functional, the enzyme is membrane bound, but appears first to be synthesized in soluble form, then incorporated into the membrane (305). Quantity of nitrate reductase formed is directly related to rate of growth. This is demonstrated particularly well by exposure to cysteine of cells growing by nitrate respiration in minimal medium, which slows both growth and the production of membrane-located enzyme simultaneously (10). Cysteine does not diminish the activity of nitrate reductase once it is formed.

During anaerobic, nitrate-reducing growth of *E. coli* Hfr H(thi$^-$) in glucose-thiamine minimal medium, the addition of molybdate stimulates synthesis of nitrate reductase. Addition of selenite along with molybdate is then necessary for the synthesis of formate dehydrogenase that serves as electron donor for nitrate reduction. These metals are not required for synthesis of other, related enzymes (74, 183).

Although nitrate respiration proceeds at the expense of a number of different electron transport phenomena in *E. coli*, it still shares one similarity with oxygen respiration. Catabolite repression of β-galactosidase synthesis is exerted by sugars and polyols in growing cells respiring in either manner, but not in cells growing fermentatively (60). Earlier attempts to demonstrate a possible organic nitrate derivative as the entity actually reduced by nitrate reductase were not successful (225), and since then little attention has been given this possibility. It is known, however, that pyridine I-oxide reductase from *E. coli* is distinct from nitrate or nitrite reductase (160).

Bacillus species. Nitrogen-depleted *Bacillus stearothermophilus* cells synthesize nitrate reductase more rapidly when transferred to anaerobic, nitrate-containing media than do fully nourished cells that are grown aerobically before transfer. No nitrate reductase is formed by either type of cell in the absence of nitrate (69), which is consistent with the finding that the ability of this organism to initiate nitrate respiration depends on selection of mutants from the inoculum population (68). The quantity of ni-

trate reductase that is produced is directly related to the concentration of nitrate and inversely related to the quantity of oxygen in the cultures (63). In this obligately respiratory, nonfermentative species, as in other bacteria, *a*-type cytochrome is expendable during nitrate respiration. Nondividing cells produce increased amounts of *c*-type cytochrome under anaerobiosis, whereas their *a*-type cytochrome contents remain unchanged. When nitrate is provided under anaerobiosis, however, a greater increase in cytochrome *c* occurs and is accompanied by a dramatic diminution in the concentration of *a*-type cytochrome in the bacilli (67). Nitrate-reducing cells retain 30 to 40% of their capacity for aerobic respiration and rapidly regain full aerobic capacity when aerated. Even if amino acids are provided as possible maintenance material, aeration results in the rapid destruction of nitrate reductase (68).

Different responses are seen in a *Bacillus* species that can grow anaerobically by either fermentation or respiration. *B. licheniformis* cells lyse when shifted to anaerobic incubation in nitrate-containing nutrient broth unless a period of weak aeration intervenes (302). The presence of glucose or organic acids protects against this lysis. During the period of weak aeration nitrate reductase appears in the cells and greatly increases with the onset of anaerobiosis. Unexpectedly, under anaerobiosis, synthesis of nitrate reductase proceeds at an identical rate whether or not nitrate is present. The enzyme is not degraded as rapidly upon return of the cells to aeration as is the nitrate reductase of *B. stearothermophilus*. Disappearance of *a*-type cytochrome from fermentatively growing *B. licheniformis* that are synthesizing but not using nitrate reductase seems consistent with the following hypothesis: in those bacteria that possess the capacity for both fermentative and respiratory alternatives to oxygen-consuming respiration, the disappearance of *a*-type cytochromes is a consequence of anaerobiosis and not of any particular mechanism of anaerobic respiration. Disappearance of the *a*-type cytochromes would then constitute a corollary rather than a specific consequence of nitrate respiration.

Azide and several other monovalent anions are inducers of nitrate and nitrite reductase production in *B. cereus* even though several are inhibitors of electron flow. Biochemically, the nitrate reductase from *B. cereus* lacks specificity for nitrate and reduces a variety of monovalent anions (e.g., bromate, perchlorate, and chlorate [117, 118]). Thiocyanate, bromate, cyanate, selenocyanate, azide, perrhenate, chlorate, perchlorate (119), and xanthine oxidase (120) competitively inhibit nitrate reductase in extracts of this *Bacillus*. Iodate is a noncompetitive inhibitor.

Proteus mirabilis. As in several other bacteria, nitrate reductase synthesis in *P. mirabilis* is derepressed by anoxia when vigorously aerated cultures in a minimal salts-glucose medium lacking nitrate are shifted to anaerobiosis by sparging with nitrogen. Greater quantities of the enzyme are formed when nitrate or nitrite is provided during anaerobiosis, however (56). Despite its inhibition of activity, the presence of azide during anaerobic growth of *P. mirabilis* in the absence of nitrate stimulates production of the enzyme more than 40-fold over that obtained without azide. Synthesis of cytochrome b_{559}, which is implicated in nitrate reduction, is also stimulated by anaerobic growth in the presence of azide. Production of formic hydrogenlyase is repressed by azide, and the activity of the enzyme is inhibited as well. Nevertheless, formate can still serve as electron donor because a system containing a formate dehydrogenase linked to nitrate reduction is synthesized in the presence of azide (56). In a mutant strain that is resistant to chlorate, but still able to form a low level of nitrate reductase, anaerobic growth in the presence of nitrate represses synthesis of formate dehydrogenase, formic hydrogenlyase, formate oxidase, cytochrome $b_{563.5}$, and cytochrome a_2 in addition to thiosulfate and tetrathionate reductases. Rather than the nitrate ion, a nitrate reductase system functioning at a detectable level is thus suggested as the agent affecting this type of repression, for azide reverses nitrate's repressive influence (57, 58). The influence of azide during growth needs careful interpretation in light of its dual role as inducer of nitrate reductase and inhibitor of electron transport.

Syntheses of a number of anaerobic electron transport phenomena are interrelated in this bacterium, as in other members of the *Enterobacteriaceae*. In the hierarchy of controls that regulate the various respiratory systems in *P. mirabilis*, oxygen dominates by repressing the synthesis and inhibiting the activity of all. Nitrate appears next in the order of dominance, for either its presence or the enzyme it induces represses formation of thiosulfate and tetrathionate reductases, and it inactivates thiosulfate reductase as well (55, 58).

Haemophilus parainfluenzae. A dominant position for oxygen over nitrate reduction, and for nitrate reduction over other anaerobic respiratory pathways, is seen in *H. parainfluenzae* as well as in the members of the *Enterobac-*

teriaceae previously mentioned. Nitrate reductase synthesis occurs in cultures incubated in either the presence or absence of nitrate when the quantity of oxygen is lowered to approximately 100 μM (308). Synthesis of a high concentration of *c*-type cytochrome and disappearance of cytochrome a_2 accompany production of a functional nitrate reductase system. Reductases for fumarate, pyruvate, and substrate quantities of NAD are then formed only when the oxygen tension reaches 15 μM. As expected, chloramphenicol inhibits synthesis of the several systems that substitute for oxygen-linked respiration in this bacterium, as in many others.

But, such is not the case in all bacteria. Although chloramphenicol inhibits incorporation of ^{14}C-labeled arginine into protein, the antibiotic does not inhibit (and in fact, stimulates) synthesis of nitrate reductase in *S. aureus* (283). This observation is difficult to explain in view of the well-established capacity of chloramphenicol for inhibition of the synthesis of protein generally, and nitrate reductase specifically, in other bacteria (256, 265).

Characteristics of the Enzyme. Location, assembly, and electron transport. With one exception (91), dissimilatory nitrate reductases are reported to be particle bound. Initial events in the electron transport that results in nitrate reduction make use of pyridine nucleotides, flavines, and quinones; but when oxygen is in short supply, a branch from the oxygen-terminated electron-transport chain occurs at the cytochrome *b* level (63, 138, 163, 224–226, 295, 305, 367). Different *c*-type cytochromes are produced and used to transport electrons that reduce nitrate rather than oxygen. NAD and FAD or FMN were revealed as cofactors for the electron transport leading up to the reduction of nitrate in several early studies, whereas quinones have only more recently been shown to be components of the nitrate-reducing electron transport chains. An endogenous naphthoquinone isolated from *B. stearothermophilus* stimulates nitrate reduction when added back to extracts of that organism (62). In addition, several *S. aureus* menaquinone-deficient mutants lack the nitrate-reducing capacity that is seen in wild-type strains (299); and in extracts of an unnamed extreme halophile, nitrate reducing activity is destroyed by irradiation at 360 nm and restored by addition of either vitamin K_1 or menaquinone-8 (197). Ubiquinone-8 is extractable from lyophilized membranes of *E. aerogenes* cells that are grown anaerobically on minimal media containing ammonia (to repress assimilatory nitrate reductase formation) and nitrate (to induce the elevated synthesis of the dissimilatory reductase). Extraction results in loss of capacity for nitrate reduction by the membranes. Addition of ubiquinone-6, -8 or -10 then restores 60% of the original NADH-dependent activity, whereas addition of menaquinone does not (168).

The quinone cofactors are tightly membrane bound in *E. aerogenes* and remain so even when the quinone structure is damaged. This is made obvious by the simultaneous loss of nitrate reductase activity and inactivation of the endogenous quinone that results from the irradiation at 354 nm of membranes held at 4 C, for the addition of ubiquinones does not restore capacity for nitrate reduction unless the remnants of the radiation-damaged quinone are removed. Pentane is employed for the extraction. Only then is ubiquinone-8 reincorporated into the membranes, where it restores 25% of the original nitrate reductase activity (167). In contrast, 90% of NADH oxidase activity lost concurrently as a consequence of irradiation of the membranes is restored by these procedures. It is thus possible that irradiation at 354 nm also damages the nitrate reductase protein as well as destroying the quinone.

Growth under nitrate-respiring conditions commits *E. coli* to diminished use of the tricarboxylic acid cycle. Succinic dehydrogenase and fumarase are not produced (347). Instead, the bacteria rely on glucose-generated NADH and formate (or its precursors, lactate or pyruvate) as contributors to electron transport (43). A cytochrome c_{552} is produced in quantity by *E. coli* and *Escherichia aurescens* (108), and when reduced it can be oxidized by nitrate. There was until recently (42) some question of its physiological significance. The bacteria form significant amounts of cytochrome c_{552} in anaerobic media containing little nitrate, but production of *b*-type cytochrome known to be necessary requires greater quantities of nitrate (387). Association of cytochrome c_{552} with nitrite rather than nitrate reduction has been demonstrated, and syntheses of the nitrite reductase and cytochrome c_{552} are simultaneously affected in *nirA* mutants of *E. coli* K-12 (42). Catabolic products of glucose fermentation differ from those formed during nitrate respiration by *E. aerogenes*. Hydrogen is not produced during nitrate respiration, and ethanol and formate production decreases. Carbon dioxide and acetate production increases, whereas pyruvate (which is not a fermentation product) is formed during nitrate respiration (86). Hydrogenase, formic hydrogenlyase and *a*-type cytochromes are not involved in the metabolism of these nitrate respirers.

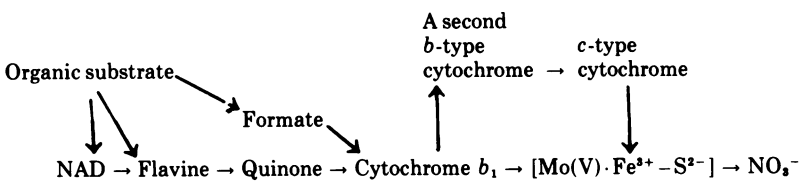
It is consistent to find, therefore, that a

formate dehydrogenase specifically linked to nitrate reduction and distinct from the formate dehydrogenase associated with the formic dehydrogenlyase system operates in *E. coli* K-12 (295) as well as *P. mirabilis* (56). Furthermore, two specific and distinct types of cytochrome b_1 that are reducible by formate and oxidizable by nitrate are functional in membrane preparations from this bacterium. *n*-Heptylhydroxyquinoline-*N*-oxide inhibits nitrate oxidation of the reduced forms of these cytochromes. The interdependence of the enzymes in this nitrate-reducing system is revealed when extracts of membranes of two mutants lacking formate dehydrogenase and cytochrome b_1-nitrate reductase, respectively, are mixed. Neither alone supports formate-linked nitrate reduction. But, when mixed together and incubated anaerobically at 37 C (378), the combination restores the capacity for formate-linked nitrate reduction. Incubation of the mixture in the cold suppresses this complementation. *E. aurescens* also produces cytochrome b_1 and synthesizes only the nitrate-linked formate dehydrogenase when grown anaerobically with nitrate (378).

An unanticipated case of nitrate reductase formation has been observed in studies of the influences of oxygen and heme precursors on cytochrome synthesis in *S. itersonii* where significant increases in production of both *b*- and *c*-type cytochromes occur at low aeration rates without concomitant increases in oxygen respiration rates (38). Twice as much *c*- as *b*-type is produced, and chloramphenicol inhibits synthesis of the cytochromes. Assays then reveal that nitrate reductase is formed in cultures that are incubated at low aeration with or without nitrate (91), but the content of a soluble *c*-type cytochrome is greater in the cells harvested from the nitrate-containing medium. The presence of nitrite also induces production of greater quantities of both nitrate reductase and the *c*-type cytochrome. Like the *c*-type cytochrome, the nitrate reductase from this organism is soluble in extracts of sonicated cells. This is the only report of a soluble respiratory nitrate reductase in a mature system and may indicate unusual fragility of the spirillar membrane rather than nonmembrane location of nitrate reductase.

For the study of those many bacteria whose nitrate reductase must be released from membranes, surfactants are useful. Deoxycholate (DOC) is superior to other detergents or salt solutions tested for solubilization of nitrate reductase from *E. aerogenes* (368). The enzyme released for investigation exists as an apparent dimer (molecular weight, 400,000; sedimentation value, 13.9*S*) convertible to a monomer (molecular weight, 200,000; sedimentation value, 8.5*S*) by DOC and nonionic detergents. DOC is not effective for solubilizing nitrate reductase from *B. stearothermophilus*. Treatment with Triton-114 does release 80 to 90% of the nitrate reductase activity from membranes of lysozyme-treated *B. stearothermophilus* (163), however. Triton X-100 is effective as well, and both release the reductase that functions preferentially with a reduced *b*-type cytochrome. Treatment with sodium dodecyl sulfate solubilizes membranes and disrupts the flow of electrons from NADH to nitrate that is mediated by enzymes in the membranes of this thermophilic bacillus (164), but the disruption is reversible. Dilution and incubation of the soluble material with divalent cations, especially Mg^{2+}, permit reaggregation of proteins and restoration of catalyzed electron flow from NADH to nitrate. Both *b*- and *c*-type cytochromes are present in both the original membranes and the reaggregated fraction. KCN and azide inhibit NADH-dependent reduction of nitrate in these preparations.

Schematically and in composite, the electron transport resulting in dissimilatory nitrate reduction in several bacteria appears to function as follows:



Pichinoty's type A reductase. Studies that distinguish nitrate reductases A and B (255) have proved useful but, as previously mentioned, leave several questions unanswered. A number of the properties of the two types of enzymes are clear and distinct, and the significance of the production of each is obvious. Other properties are not well defined, however, and the significance of the type of enzyme produced is not certain. We know, for example, that enzyme type A from many bacteria is a membrane-located, respiratory catalyst capable of reducing chlorate and bromate as well as nitrate (but not iodate). In contrast, enzyme B does not reduce chlorate but is, in fact, inhib-

ited by it. Enzyme A from *E. aerogenes* and *M. denitrificans* oxidizes NADH but not NADPH anaerobically. This activity is removed by DOC or by incubation at 55 C for 4 min and is replaceable by $FMNH_2$ or reduced viologen dyes (258).

The activity of enzyme A is sensitive to as little as 1 mM azide, whereas the activities of enzyme B and a separate chlorate-reducing enzyme C, unrelated to nitrate reductase, are much less sensitive to azide. Azide is a competitive and reversible inhibitor of enzyme A activity, whereas inhibition by cyanide is not competitive and is only partially reversible. The affinity of enzyme A for nitrate is greater than that exhibited by B (257, 269). Manometric assay for the consumption of hydrogen during reduction of chlorate or nitrate in systems containing hydrogenase and viologen dyes is used to determine enzyme activity of both enzymes A and B, although a colorimetric method for the determination of nitrite production by enzyme A is recommended as well (268). In a number of enteric bacteria, azide and nitrite (37) serve as inducers of enzyme A synthesis, but azide does not induce synthesis of enzyme B.

When a broad spectrum of nitrate-reducing bacteria is assayed for these enzyme types, few clear inter- or intrageneric distinctions are made apparent. Several members of the *Enterobacteriaceae* produce enzyme A only, but the family cannot be characterized exclusively as producers of enzyme A. Other members produce enzyme B and not A, and some produce both (263). Included among those that form A only are *E. coli*, representatives of the Alkalescens-Dispar group, and members of the genera *Arizona*, *Citrobacter*, *Enterobacter*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, and *Shigella*. In contrast, *Edwardsiella* and *Hafnia* species produce only enzyme B (except for one *Hafnia* strain that forms both A and B). The *Providencia* species present interesting contrasts. Seventeen *P. alcalifaciens* strains all produce enzyme B but not A. In contrast, 11 of 13 *P. stuartii* strains produce A but not B, one produces B but not A, and one produces both. Among the pseudomonads assayed, *Aeromonas* species produce enzyme B only. Several species of *Pseudomonas* form enzyme A, others B, and a few both. *Thiobacillus denitrificans* and *Micrococcus halodenitrificans* (262) synthesize enzyme A, *Yersinia* species enzyme B, and *M. denitrificans* enzyme A and B. In the genus *Bacillus*, enzyme A predominates with 21 strains representing 10 species producing enzyme A. But, even in this genus, one strain in each of two species forms B and no A. A *Vibrio* species that grows anaerobically at the expense of nitrate nitrogen in the presence of chlorate is assumed without testing to form only enzyme B on the grounds that bacteria that produce enzyme A reduce chlorate to toxic chlorite and thus cannot grow in its presence.

Despite the emergence of no clear pattern of distinction between genera or species that produce one or the other enzyme, there are a number of dependable and helpful observations. It is clear that enzyme A serves a respiratory function in every bacterium that produces it. It is consistent, then, that the nitrate reductase of all denitrifiers tested is type A. Enzyme B obviously carries out an assimilatory function in several bacterial species that form B constitutively or grow aerobically with nitrate as sole nitrogen source. But, several species that do not produce enzyme B nonetheless assimilate nitrate nitrogen. Moreover, in a number of species, production of enzyme B is repressed by oxygen. This has been taken as an indication that enzyme B may have a respiratory role in these bacteria, but follow-up assays for possible assimilation of labeled nitrite generated by such an enzyme or esterification of phosphate accompanying the action of a B-type enzyme have not been carried out. It is also possible that enzyme B is produced by more species than currently is realized. Assay of enzyme B in the presence of a large quantity of enzyme A is difficult, and for this reason enzyme B activity may be overlooked.

It seems likely that the best prospect for progress toward clarification lies with intensive biochemical studies of purified enzymes. Enzyme A released from *M. denitrificans* particles by alkali-acetone treatment has been purified 50-fold to 95% homogeneity by gel filtration and column chromatography (84). Molecular weight is estimated at 160,000, and no flavine is associated with the isolated protein, but $FMNH_2$ and $FADH_2$ will serve as electron donor. This distinguishes enzyme A from fungal assimilatory nitrate reductase which is a molybdoflavoprotein (64). Reduced viologen dyes and free $FADH_2$ and $FMNH_2$ serve as electron donors for nitrate reduction by purified enzyme A; NADH and NADPH do not. The protein is rich in aspartate and glutamate but poor in cysteine.

Several investigations recently carried out provide insights into the function of metals in nitrate reductase A which contains molybdenum, as previously suggested in 1961 by work with a cruder preparation (82) and nonheme iron. EPR measurements at 80 K reveal Mo(V) in the purified enzyme; and at 15 K, Fe(III) is observed in enzyme A from *M. denitrificans* (85). Reaction with nitrate, nitrite, or azide

alters the Mo(V) signal. Reduction of enzyme A either chemically with dithionate or enzymatically with hydrogenase and molecular hydrogen results in disappearance of the molybdenum and iron signals. Electron flow apparently proceeds from *b*-type to *c*-type cytochrome, from there to Mo(V), on to Fe(III), and thence to nitrate. Similar studies with purified preparations of both an oxygen-sensitive and oxygen-insensitive enzyme B would be useful now to provide the bases for comparing the two functional types of enzyme, A and B.

Genetic Studies. The observation that several bacterial species incubated in the presence of perchlorate lose the ability to reduce nitrate (121) led several investigators to search for mutants with altered nitrate respiration. Their studies revealed that, when grown anaerobically, a variety of enzyme A-producing enteric bacteria can reduce chlorate to chlorite in toxic quantity. Possession of the capacity for producing enzyme A therefore prevents their growth under anaerobic conditions in the presence of chlorate (11, 270, 272, 330). The chlorate-resistant mutants (ChlR) of *E. coli* and other members of the *Enterobacteriaceae* thus lack nitrate reductase A; and in the overwhelming number of isolates, chlorate reductase C and formic hydrogenlyase are missing as well. Many reveal pleiotrophic effects by the simultaneous loss of enzyme B, assimilatory nitrite reductase, tetrathionate reductase, and thiosulfate reductase (272). This simultaneous loss of several properties is reflected by disappearance of a large protein fraction from sedimentation profiles of *E. coli* membrane particles (11). Mutant strains of *E. coli* K-12 and certain of the *E. aerogenes* mutants lack formate dehydrogenases as well; but, in general, cytochrome components, hydrogenase, and fumarate reductase remain unaffected in ChlR strains (264, 331, 333, 373). *E. coli* K-12 mutants showed close association between levels of formate dehydrogenase, nitrate reductase, and cytochrome b_1. These mutants reverted to wild type with a return of wild-type level for each of these (296). Revertants are discernible by the return of measurable capacity for anaerobic reduction of nitrate or chlorate in facultative bacteria, use of nitrate as sole nitrogen source in strictly aerobic bacteria, and liberation of nitrogen from nitrate in bacteria capable of denitrification (271).

Chlorate resistance and the various characters associated with it are transducible in the enteric bacteria by several phages (2, 208, 278, 372). Thus, using assays for co-transduction frequency of ChlR with known markers, the genetic locus for its control was first mapped between the locus for galactose utilization (*gal*) and that for biotin synthesis (*bio*) on the *E. coli* genome (278, 351). But the high frequency of mutation indicates polygenic control, as evidenced by the three sites that occur in *E. aerogenes* (334). In *E. coli* four sites are now mapped (279); *chlA* near *bio*, *chlB* between the genes for isoleucine-valine synthesis (*ilv*) and one of the loci controlling methionine synthesis (*metE*), *chlD* between *gal* and the gene controlling production of the attachment site for phage λ(*att*λ), and *chlE* between *chlA* and purine synthesis gene (*purB*) some distance away. The few ChlR isolates (1%) that retain formic hydrogenlyase are designated C, and *chlC* maps near the tryptophan-synthesis gene (*trp*) between *purB* and the gene for the synthesis of the attachment site for phage θ80 (*att*θ80). The genetic determinant for nitrate reductase transduced by phage PLT_{22} among *Salmonella typhimurium* mutants and wild-type controls tetrathionate reductase, formate hydrogenlyase, and formate dehydrogenase as well (208).

Following exposure to a mutagen, the method of mutant selection employed influences the complementation groups derived from populations. For example, nearly 50% of the nitrosoguanidine-mutated *E. coli* strains selected for inability to link nitrate reduction to formate oxidation are in the *chlC* class as opposed to 1% of those selected for ChlR (96, 350). The mutants chosen in this new fashion exhibit two new *chl* loci (*F* that maps near *trp* and *chlC*, and *G* near 0 min).

The physiological consequences of the functioning of *chl* genes are quite varied. The gene product of *chlB* is necessary for production of the particle lost from the sedimentation profiles of membranes from mutant cells (32), and transduction of *chlA* into polygenic mutants restores to them the capacity for synthesizing several proteins in the particles (293). Mixture of extracts from *chlA*, *B*, and *C* grown separately with nitrate provides the enzymes necessary for nitrate reduction at the expense of reduced viologen dyes (277). In vitro complementation is reportedly carried out with a mixture of cell-free extracts from *chlA* and *chlB* mutants yielding soluble enzyme that reduces nitrate and chlorate at the expense of reduced viologen dye or reduced flavines. The enzymes are said to become particulate in time by aggregation with phospholipid and cytochrome b_1 (8, 9). *chlB* mutants of *E. coli* produce material recoverable from the cytoplasm that complements the *N. crassa nit-1* product after acid treatment and restores nitrate reductase activity (193). This material does not complement products of other *E. coli* mutants. High concentrations of molybdate in the growth me-

dium restore the capacity for synthesis of both formate-linked nitrate reductase and formic hydrogenlyase activity in *chlD* mutants. This was interpreted as indicating that the gene product of *D* thus exerts an indirect influence on nitrate reductase synthesis by contributing to processing molybdate to the form in which it functions in electron transport (95). Selenite is then required for synthesis of the enzymes that enable formate to serve as electron donor for the nitrate reductase of these mutants (183). It is now apparent that *chlD* controls synthesis of two membrane components whose production is induced by anaerobic growth in the presence of nitrate. Locus *chlA* specifies the constitutive synthesis of three proteins involved in nitrate respiration (294).

Rather than screening for ChlR, Venables and Guest devised and employed a lactate-nitrate medium for direct selection for *E. coli* K-12 mutants which have lost the capacity for producing nitrate reductase (372). Controlling loci are thus designated *nar*. The *nar*(*chl*)*C* locus of mutants derived in this fashion still maps within the limits previously mentioned. But, more specifically, it lies between the heme-deficiency (*hemA*) and the ochre suppressor genes (*supC*) and appears to represent the structural gene for nitrate reductase (114). Closer investigation of mutants classed as *nar*(*chl*)*A*, *B*, and *E* reveals that each represents more than one complementation group and, furthermore, that each may comprise more than one gene (117). Regulation of nitrate respiration thus appears more complex than seemed likely when ChlR was first isolated.

Presumably, a large fraction of aerobically grown cells with a genotype conferring the ability to reduce nitrate are transformed to nitrate reducers by removal of repressing oxygen and the presence of nitrate. But, mutation accounts for the ability of one species to generate a population that can carry out this type of anaerobic respiration. Nitrate-reducing mutants of *B. stearothermophilus* are selected from wild-type populations by lowered oxygen tension and the presence of nitrate (68). Fluctuation tests reveal mutations occurring at 7.5×10^{-8} per cell per generation, as opposed to conversion of an average of 82% of the viable cells in aerobic populations of *Pseudomonas perfectomarinus* to nitrate respiration upon anaerobic incubation in nitrate-containing medium (254). Nitrate reduction is repressible by oxygen even in the *B. stearothermophilus* mutants (as it is in other bacteria), but constitutive mutants of the *Bacillus subtilis* Marburg strain are obtained from populations treated with ethyl methyl sulfonate (113). This mutation is pleiotrophic and results in concurrent asporogenesis and loss of other properties.

In *B. licheniformis*, 36 single-site mutations used in transformation and transduction experiments indicate that eight ChlR groups are discernible (303). The protein patterns obtained by gel electrophoresis of membranes from the groups of mutants thus established differ greatly from the patterns obtained upon electrophoresis of the proteins from wild-type cell membranes.

ChlR strains of the nonfermentative bacterium, *P. aeruginosa*, are assigned to five *chl* (or *nar*) complementation groups (365). All lack nitrate reductase A and two lack B as well. As in the *chlD* mutant of *E. coli* (95), growth in the presence of high concentrations of molybdate restores the capacity of one of the *P. aeruginosa* mutants (*narD*) to reduce nitrate. Using interrupted mating, *nar* loci *B* and *C* are mapped near *leu-1*, but the positions of *narA* and *D* are not certain. *narC* maps quite distant from the others near *ilv*.

In addition to these ChlR (or Nar) groups in *P. aeruginosa* there are five groups of mutants (Nir) lacking the capacity for growth at the expense of nitrite respiration (364). *nirA* and *B* map near *met* and *trp*. Nitrite is actually reduced by strains in two of these Nir groups, but even so, the mutants will not grow anaerobically with nitrite as electron acceptor. This observation is consistent with the hypothesis that only the membrane-bound nitrate and nitrous oxide reductases may be coupled to phosphorylation. If, as suggested earlier (254), reduction of nitrite and nitric oxide by soluble enzymes is not linked to phosphorylation, the *P. aeruginosa* mutants that do not grow when supplied with nitrite may lack the capacity for the production of either nitric oxide or nitrous oxide reductase. The inability either (i) to generate nitrous oxide by reduction of nitric oxide or (ii) to carry out phosphorylation linked to reduction of nitrous oxide once it is formed would explain equally well the inability of these mutants to grow at the expense of nitrite.

Use as a Marker or Reagent. Assay of nitrate reductase activity has been usefully applied in a number of practical and ingenious ways. In a diagnostic laboratory, for example, Washington and Yu (384) employ nitrate reductase, deoxyribonuclease, and catalase as constant and specific markers of *S. aureus* in their evaluations of reagent-impregnated strips for determining coagulase and mannitol fermentation for differentiating micrococci. Nitrate reductase is one of a number of enzymatic activi-

ties that characterize the presumably pathogenic *Rettgerella* strains isolated from urinary tract infections (83).

Tagging membrane fractions with nitrate reductase is useful as well. In his study of the proteins of *E. coli* cell walls and membranes, the certainty of the association of dissimilatory nitrate reductase with membrane leads Schnaitman (300) to identify this activity in fractions to assure their originating in the membrane. Fractions not containing the activity are identified as cell wall in origin. Nitrate reductase is also useful, along with permeases, as an indicator of the segregation of membrane properties in induced *E. coli* inocula multiplying under noninducing conditions (6).

Because chemical reductants may generate nonspecific substances that interfere by reacting with the nitrite detection reagent, enzymatic assays for nitrate contents of soil and plant extracts and water are reportedly more specific and reproducible than chemical methods. *Rhizobium* bacteroids in soy bean nodules provide a source of enzyme for such quantitative assays (189). In addition, assay of the activity of *R. japonicum* bacteroids provides a value that correlates directly with the host plant's total nitrate reductase capacity and is used as an index of the nitrogen-fixing efficiency of the bacteroids (18). Another quantitative procedure for the assay of nitrate concentrations as low as 0.01 μg/ml makes use of formate-nitrate reductase from *E. coli* B as reagent (206). And, recently, tests for nitrate reductase were found useful in screening for possible bacterial infection of industrial-scale citric acid fermentation of molasses by *A. niger* (173).

Nitrite, Nitric Oxide, and Nitrous Oxide Reductases

Gas Chromatographic and Isotopic Methods of Analysis. For lack of an adequately long-lived radioisotope of nitrogen to serve as a tracer, many earlier biochemical studies of the reduction of nitrite and the nitrogenous intermediates depended on manometric assays. These investigations provided much useful information on the preliminary enzymatic events that initiate electron flow and identified pyridine nucleotide and flavines as cofactors for the initial events (224, 225), but their necessary reliance on manometry denied the investigators the precision needed for accurate identification and repetitive demonstration of the sequential production and reduction of the gaseous intermediates.

As an alternative, mass spectrometric assays for products of $^{15}NO_3^-$ reduction may be useful, but these procedures serve well only in experiments with narrow scope and limited objectives. Care is needed in the interpretation of data derived from studies of ^{15}N-labeled nitrate reduction, for bacteria preferentially reduce $^{14}NO_3^-$ at instantaneous fractionation rates of 1.03 under certain conditions (386). Nonetheless, sure identification of ^{15}N-labeled nitrous oxide has provided convincing evidence that this gas is an intermediate produced and utilized during denitrification by *Pseudomonas denitrificans* (225). In ecological studies (99, 101), mass spectrometry has been employed to reveal the release of heavy nitrogen by microbial reduction of ^{15}N-labeled nitrate as a means of establishing that denitrification does take place in natural waters. And, in a decisive experiment, Hart, Larson, and McCleskey (124) employed similar assays to show that nitrous oxide is the terminal product of denitrification by *Corynebacterium nephridii*. But, despite the useful outcomes of these experiments, the uncertainty of the identity of the gases assayed by manometry and the expense and technical demands of mass spectrometry combine to emphasize the need for a method that provides the means for the simple, precise, and repetitive analyses that are required for detailed investigations.

Gas chromatography is an obvious choice, even though initial, well-conceived attempts by Smith and Clark (309, 310) to determine denitrification in soils were unfortunately undertaken before their time. These workers had no column packing materials capable of resolving the gases of interest. In 1966, however, Hollis (132) introduced porous polyaromatic beads for the chromatographic separation at room temperature of nitrogen, oxygen, nitric and nitrous oxides, and carbon dioxide. Helium is employed as carrier gas and thermal conductivity for detection. Barbaree and Payne (12) then devised methods for using columns packed with these beads in their assays for products of denitrification by *P. perfectomarinus*. Their studies reveal that cells growing in complex medium at the expense of either nitrate or nitrous oxide release nitrogen but none of the nitrogenous intermediates, whereas both nitric and nitrous oxides are produced and reduced in anaerobically incubated reaction mixtures containing cell-free extracts of nitrate-grown cells (12, 252, 253). Standard curves for quantitation are obtained by integrating the areas under peaks traced by a recorder for a series of known quantities of each gas. Subsequently, gas chromatography has served several workers for determining (i) that nitric oxide is a specific

product of nitrite reduction, (ii) that nitrous oxide results from nitric oxide reduction, and (iii) that nitrous oxide is the terminal denitrification product of several bacterial strains (110, 202, 254, 284, 285).

Development of procedures for field investigations of denitrification are particularly complex. Simultaneous elaboration and testing of both sampling techniques and assay methods are required and are simultaneously complicated by a need for anaerobiosis throughout the experimental periods. Guiraud and Berlier (116) designed an incubator for soil cultures supplied with ^{15}N-labeled nitrate. From the atmosphere of the chamber above the soil samples, gases may be drawn and assayed for isotope excess of $^{15}N_2$ as a measure of denitrification. In other studies of soils (61), gases obtained with a device employed for in situ sampling of field soils are chromatographed. Ethylene production (presumably microbial) is taken as an indication of anaerobiosis, and nitrous oxide evolution as evidence of denitrification. In a series of equipment-testing experiments, activity in Berkshire soil was greatest during the wet winter months. Finally, for ecological studies of denitrification in forest soil and salt marsh mud, Todd and Nuner (359) and Payne (251) report effective use of a conical sampler that is set firmly over the test soil or sediment, flushed with helium, pulsed with neon and either nitrous oxide or nitrate, and sampled at intervals for changes in the N_2-Ne ratio. (Good practice dictates use of an invariant internal comparator standard for quantitation by gas chromatography in experiments of this sort; and neon, which emerges distinct from and ahead of nitrogen on the columns used, is an effective and reliable nonmetabolized gas for this purpose.)

Control of Enzyme Synthesis and Activity. It has been known for some time that the rate of synthesis of the denitrifying enzymes is inversely related to the availability of oxygen in all bacteria capable of denitrifying respiration (44, 225). This relationship prevails upon production of the enzymes (i) by the many bacteria that reduce nitrate to nitrogen, (ii) by those few that do not reduce nitrate but reduce nitrite to nitrogen (34), and (iii) by the small number that reduce nitrate to nitrous oxide but no further (110, 124, 285). Nitrate concentrations of 0.1 to 0.5% and nitrite concentrations up to 0.05% are optimal for growth of several denitrifying soil isolates in various types of media (19). Supplied in unrestricted quantities, nitrous oxide supports good growth of others (199, 253). Incubation temperatures near 30 C and pH values at or slightly above neutrality generally favor denitrifying growth of these bacteria. Thermophilic denitrifying bacilli that grow at 55 C are known (68, 69), but in a denitrifying psychrophilic pseudomonad with a growth optimum at less than 20 C, incubation at temperatures as high as 24 C prevents nitrite reduction (172). It is thus meaningless to specify optimal conditions for the activity of denitrifying enzymes without indicating the origin of the enzymes.

As carbon and energy sources, the usual array of carbohydrates, organic acids, or the organic components of complex culture media support denitrifying growth for various bacteria (81, 87, 94, 286). Certain aspects of aerobic catabolism of carbon compounds change when the bacteria are grown under denitrifying conditions, whereas others remain unchanged. Mixed cultures enriched from sewage sludge make use of glycolysis, the pentose phosphate shunt, and the tricarboxylic acid cycle and produce elevated quantities of *c*-type cytochromes while growing as denitrifiers (72). Radiorespirometric studies show that glucose and gluconate are catabolized by the Entner-Doudoroff and pentose phosphate pathways in *Pseudomonas stutzeri* growing either aerobically or at the expense of denitrification. The tricarboxylic acid cycle operates during both types of respiration, but release of carbon dioxide is slower during denitrification (321). Valine, leucine, isoleucine, and cysteine are incorporated from the culture medium by *P. stutzeri* during aerobic growth, but not during nitrate respiration (248). Asparagine is one of several simple organic compounds that support aerobic growth of *P. perfectomarinus*, but represents the only one of many tested that will serve as sole source of carbon and energy for denitrifying growth (286). Once transported, the asparagine is transformed to malic acid, which is the true electron donor and supplier of carbon skeletons for the growth of this marine bacterium despite its inability to serve as the sole carbon and energy source when supplied exogenously (17).

Growth substrates such as linear hydrocarbons and ring compounds that are generally assumed to require oxygenation by free oxygen during catabolism can serve, nonetheless, as sole carbon and energy sources for the denitrifying growth of several pseudomonads (353, 361). Protocatechuic acid-4,5-oxygenase breaks aromatic rings during aerobic growth of one pseudomonad, but ring cleavage by a different pathway in which benzoate is the probable key intermediate is postulated for anaerobic growth in nitrate-containing medium. Labeled carbon dioxide is released when this bacterium is incubated anaerobically with ^{14}C-ring-labeled

benzoate and either nitrite or nitrous oxide (352). It is reasonable to hypothesize that oxygenation occurs by hydroxylation during anerobic respiration, but experiments with $H_2{}^{18}O$ have not been reported. Oxidizable inorganic electron donors serve the obligately chemolithotrophic bacterium *T. denitrificans*, which can reduce nitrate to nitrite, nitric oxide, nitrous oxide (1, 355), and nitrogen (137) during growth. Sulfide, sulfite, thiosulfate and hyposulfite are utilizable electron donors, and the influence of inhibitors suggests that cytochromes are involved in the sulfite-dependent denitrification mechanisms of this organism (1). In view of the reports that sulfite reductases in other bacteria reduce nitrite as well (156), the oxidation of sulfite by nitrogenous oxides in this organism seems all the more remarkable.

Several investigators have been interested in identifying the initiator of synthesis of the denitrifying enzymes. The nonfermentative denitrifying bacteria can grow anaerobically only if supplied with an inorganic substitute for oxygen. Nevertheless, there are several indications that lack of oxygen rather than presence of the electron acceptor has the more decisive influence on reductase synthesis. It may be generally stated that production of all the denitrifying enzymes is derepressed by anoxia or lowered oxygen tension. Nitrous oxide reductase is less strongly repressed by oxygen than the other reductases. This is well illustrated when aerobic cultures of *P. perfectomarinus* (254) and *P. denitrificans* (199) are permitted to go suddenly anaerobic in the absence of any of the nitrogenous oxides. Nitrous oxide reductase synthesis begins first and reaches maximum within 3 h, whereas synthesis of nitrite and nitric oxide reductases is significant but less strong during the 3-h period. Growth of these bacteria at the expense of nitrous oxide reduction also yields cells with elevated nitrous oxide reductase activity and measurable, but even weaker, reductase activity for nitrite and nitric oxide (199, 253, 254).

Initiation of synthesis from the "other end" (i.e., with nitrate present when anaerobiosis is effected) does not provide an advantage for production of any one of the denitrifying enzymes, however. When cells are harvested from fully aerobic cultures and incubated anaerobically with nitrate, synthesis of all the denitrifying enzymes begins simultaneously within 40 min and reaches maximum in 60 min (253). This observation is seemingly at odds with the accumulation of a large fraction of the nitrate nitrogen as nitrite prior to the onset of extensive gas release in denitrifying cultures (358), in seawater (98), and in lake sediment (36). Accumulation of nitrite thus appears at first glance to argue for sequential induction (under oxygen-poor conditions) of nitrate reductase by nitrate, followed then by induction of nitrite reductase by the accumulating nitrite—as the apparently later onset of nitrite than nitrate reductase synthesis in *M. denitrificans* cultures suggests (177). But that possibility is obviated for *P. perfectomarinus* by the observation that, in the presence of nitrate and the absence of oxygen, all the denitrifying enzymes are demonstrable simultaneously. Operation of regulatory mechanisms that rank order the nitrogenous oxides for reduction seriatim by influencing the enzymes' activities rather than the sequence of their production is thus suggested. This notion is supported by studies of the effect of nitrate on the specific reducing activity of fractions separated from crude extracts of *P. perfectomarinus* (253, 254). One complex fraction reduces only nitrite, another only nitric oxide, and the other only nitrous oxide (with NADH or malate-NADP-NAD in combination serving as electron donor and free flavines acting as intermediate carriers for each). The presence of nitrate influences the rate of activity of the nitric oxide reducing fraction only and not the activity of either of the other reductases. The suppressive influence of nitrate is curvilinear from 0 to 1 μmol/ml, at which concentration the activity is diminished by 60%. Greater quantities of nitrate do not further suppress activity. Nitrite also suppresses nitric oxide reduction, but five times as much is required to achieve the same degree of suppression; and as is true with nitrate, increased quantitites of nitrite do not further suppress activity (253). Lactate-dependent reduction of nitric oxide in crude extracts of *P. denitrificans* is also suppressed markedly by nitrate (209). The significant result of this fine control exerted by nitrate and the grosser control displayed by nitrite is insurance that the ionic oxides are depleted before the gaseous ones.

Electron Transport Cofactors. In the flow from substrate to the cytochrome level, the electron transport events that lead to reduction of nitrite, nitric oxide, and nitrous oxide are unexceptional in the various denitrifying bacteria. A variety of oxidizable substrates are utilized in culture or cell suspensions, and NADH or reduced free flavines serve as donors for enzymes in cell-free extracts (82, 177, 224, 225, 382). In *P. perfectomarinus* (17), a Mn^{2+}-dependent, NADP-linked malic oxidative decarboxylase initiates electron flow, but transhydrogenase links electron flow to NAD and free flavines. Transport by natural cofactors has not been observed in every case. In a number of

experiments with cell-free systems, artificial or exotic electron transfer reagents are required (281). Denitrification carried out by extracts from *M. denitrificans* at the expense of D- or L-lactate proceeds if beef heart cytochrome *c*, 2,6-dichlorophenol indophenol, or phenazine methosulfate (PMS) is supplied, but provision of pyridine nucleotides has no effect on activity (244). In particulate form, the denitrifying reductases from *P. denitrificans* use NADH, but when solubilized with DOC, they require reduced viologen dyes (282).

Interactions between certain electron donors and the nitrogenous oxides result from both chemical and enzymatic activities. Gas is released by the reduction of nitrite by an enzymatic fraction from acetone powders or cell-free extracts of *P. denitrificans* by using hydroxylamine or either tetramethyl- or dimethyl-*p*-phenylene diamine as electron source (141, 211, 344, 345). Identification of nitric oxide in the gas is thought to strengthen the case for this compound as an intermediate of denitrification, although the complex chemical reactions that occur in systems supplied with hydroxylamine or the diamines make interpretation of this type of reduction difficult. For example, nitric oxide reacts with the phenylene diamines to form nitrogen nonenzymatically. Moreover, hydroxylamine inhibits nitrite reduction (200), suppresses reduction of nitric oxide, and together with nitric oxide suppresses reduction of nitrous oxide (198). The nitrogen released by reduction of nitrite at the expense of one of the phenylene diamines by cell-free systems derived from *M. denitrificans* and *P. aeruginosa* apparently combines one nitrogen atom from nitrite with another contributed by the diamine (259). Hydroxylamine's involvement in the truncated denitrification carried out by extracts of *C. nephridii* is similarly complex and difficult to assess (284). What relation, if any, these various reactions have to true denitrification is not apparent.

Indirect evidence of the participation of cytochromes in denitrification is provided by the simultaneous isothermal bleaching of the cytochromes by strong light and death of *M. denitrificans* populations growing under denitrifying conditions (123). Furthermore, the manner in which toxic compounds inhibit the sulfite-dependent denitrification carried out by *T. denitrificans* suggests the involvement of cytochrome (1). And, finally, growing cultures of *P. aeruginosa* and *P. denitrificans* provided with Δ-amino levulinic acid accumulate greater quantities of the cytochrome precursors, protoheme and protoporphyrin, under anaerobic, nitrate-respiring than under aerobic conditions (142).

More direct evidence for the participation of cytochromes is provided by isolation from *P. denitrificans* (209), *P. aeruginosa* (382), *Alcaligenes faecalis* (200, 201), *Achromobacter fischeri* (276), and *M. denitrificans* (177, 228) of *b*-, *c-d*-, and *c*-type cytochromes that influence the rates at which gas is released by reduction of one or more of the nitrogenous oxides. Association of *a*-type cytochromes with denitrifying preparations (178, 228, 388) apparently occurs by chance proximity in the membrane. Anaerobic, nitrate-dependent growth of *M. denitrificans* represses synthesis of the $a + a_3$ cytochromes characteristically produced by well-aerated cells and by those grown at low aeration in the absence of a nitrogenous oxide (298, 301). Growth at low aeration or under anaerobic, denitrifying conditions yields a *c-d*-type cytochrome not produced by fully aerated *M. denitrificans* cells. When grown anaerobically with nitrate serving as terminal oxidant, *R. japonicum* also ceases to produce cytochrome $a + a_3$. Moreover, increases in the production of *c*-type cytochrome, *Rhizobium* hemoglobin, and cytochrome P450 are concomitant with increases in the synthesis of nitrate and nitrite reductases in these cells (53).

Although none has been isolated and completely characterized, several cytochromes have been associated with reduction of specific nitrogenous oxides. When reduced chemically, a cytochrome c_{551} from *A. fischeri* and a double α-peaked *c*-type cytochrome from *P. perfectomarinus* are oxidized by nitrite to a state that exhibits the oxidized spectrum (50, 276). Two double α-peaked *c-d*-type cytochromes have been isolated from nitrite reductases and studied extensively. That produced by *M. denitrificans* has an apparent molecular weight of 120,000, whereas that recovered from *P. aeruginosa* has a molecular weight of 85,000 (228, 229). A *c-d*-type cytochrome associated with nitrite reduction by *A. faecalis* has a molecular weight of 90,000 (139). *P. denitrificans* produces a copper-containing cytochrome c_{552} or c_{553} that couples lactate oxidation to the reduction of nitrite to nitric oxide (199). This enzyme complex has oxygen-consuming and hydroxylamine-oxidizing capacity as well and utilizes a variety of artificial electron donors in the reduction of nitrite (212). *A. faecalis* yields a *c-d*-type cytochrome that participates in the reduction of nitric oxide to nitrous oxide in cell-free extracts supplied with

ascorbate-reduced PMS as electron donor (201).

In one instance, nitrite reduction does not appear to involve flavine or cytochromes. A copper-containing enzyme from *Achromobacter cycloclastes* utilizes ascorbate-reduced PMS or hydroxylamine as electron donor, it is suggested, without mediation by electron transport cofactors (140).

Ideally, ambiguity as to which of the denitrifying steps the various cytochromes are contributing to can best be eliminated by working, one at a time, with systems that reduce each of the nitrogenous oxides only to the next intermediate or to the final product. Payne and his colleagues were fortunate therefore to find that each of three complex fractions separated from crude denitrifying extracts of *P. perfectomarius* specifically reduces only one of the identified intermediates of nitrate reduction (253, 254). Recently, a cytochrome c_{548} isolated from the fraction that reduces nitrite to nitric oxide has been found to stimulate the rate of reduction when added back to a system containing NADH, free flavine, extracted enzyme, and nitrite (50). EPR measurements indicate no metal involvement, but do reveal that a heme-nitric oxide complex is formed while nitric oxide is evolving from the reaction mixture during active reduction of nitrite. A second fraction exhibiting a different, tightly bound *c*-type cytochrome reduces nitric oxide to nitrous oxide also at the expense of NADH. EPR assays again reveal no metal involvement, but indicate the formation of a different heme-nitric oxide complex during active release of nitrous oxide (51). The EPR signal broadens with time as reduction of nitric oxide continues and nitrous oxide accumulates. A third, membrane-bound fraction reduces nitrous oxide to nitrogen but has not been analyzed for cytochrome content. Denitrification in *P. perfectomarinus* may be diagrammatically characterized as indicated in Fig. 1.

The retention of the nitrous oxide reductase complex by the membrane fraction from *P. perfectomarinus* when ruptured cells are centrifuged may explain the inability of extracts of other denitrifying bacteria that can reduce nitrite and nitric oxide also to reduce nitrous oxide after centrifugation (200, 202, 210, 253, 254).

Curiously, the ammonia-oxidizing bacteria that ordinarily generate nitrite can also reduce it. *Nitrosomonas europaea* contains enzymes that catalyze hydroxylamine-dependent reduction of nitrite to nitric oxide and nitrous oxide (133, 390). The significance of this phenomenon

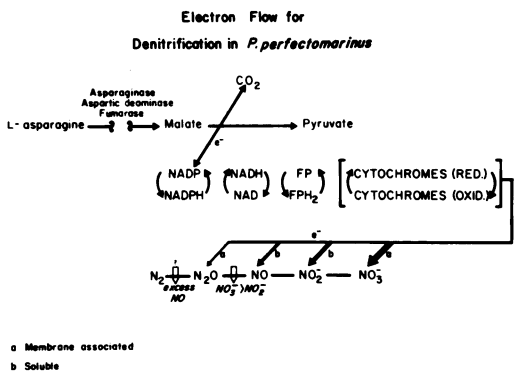


FIG. 1.

is not yet known, although its use by the ammonia-oxidizing bacteria as a temporary aid to survival during periods of anaerobiosis is suggested (292).

Attendant Conservation of Energy

Dissimilatory nitrate reduction and denitrification are energy-yielding types of respiration, but the points of coupling of electron transport and phosphorylation have not been determined. Of the four oxidants that have been identified as components of the denitrification sequence, it is intuitively obvious that bacteria respiring anaerobically at the expense of nitrate and nitrous oxide must couple their reduction with phosphorylation. One-step reduction of each of these compounds (to nitrite and nitrogen, respectively) is known to support the growth of various bacteria (199, 225, 253). But, those that grow when supplied with an exogenous source of nitrite or nitric oxide, which they reduce finally to nitrogen, may conceivably benefit from coupling reduction with phosphorylation only during the last step (i.e., nitrous oxide reduction). If it is further assumed that adenosine triphosphate (ATP)-generating respiratory systems remain membrane associated when cells are ruptured, then nitrate and nitrous oxide reductases qualify as such (6, 305). But, nitrite and nitric oxide reductases are solubilizable (50, 254) and thus may not represent phosphorylating systems.

There are yet no reports of oxidative phosphorylation coupled specifically to nitrous oxide reduction, but several studies correlating nitrate reduction and phosphate esterification have been carried out. In an early experiment, particles and supernatant portions of extract from nitrate-respiring *P. aeruginosa* (389) were separated by centrifugation and tested individually for capacity to esterify P_i. But, neither

fraction was effectual alone, and ATP generation occurred only when the two were combined and were oxidizing lactate and reducing nitrate. The P-NO_3^- ratio was 0.3. Later, sonic extracts of nitrate-adapted *P. denitrificans* were found to synthesize ATP as a result of electron flow from succinate to nitrate (235). Esterification of ^{32}P-labeled phosphate is revealed by the enzyme-mediated, ATP-dependent formation of glucose phosphate. Because ATP may be dissipated by competing reactions, this trapping procedure provides an easily assayed but specific indicator of synthesis. The concentration of ADP controls the rate of both nitrate and oxygen consuming respiration in the extracts.

Esterification of ^{32}P-labeled phosphate concomitant with reduction of nitrate to nitrite by extracts of sonicated *E. coli* cells has also been demonstrated with a variety of organic acids or NADH serving as electron donors (237). A mixture of soluble and particulate fractions is required as mentioned before. P-NO_3^- ratios range with electron donor from 0.23 to 1.1. Protein fractions necessary for coupling phosphorylation to reduction of either oxygen or nitrate have been isolated from extracts of sonicated cells (236). Ultraviolet irradiation destroys the coupling capacity of these fractions. 2,4-Dinitrophenol (DNP) inhibits phosphorylation of the protein in the fractions, but does not prevent transfer of phosphate from previously phosphorylated protein to ADP.

Phosphorylation coupled to nitrate and nitrite reduction by a particulate fraction from *M. denitrificans* is also achieved with organic acids or NADH serving as electron donors (135, 223). There is no esterification in reaction mixtures supplied with nitric oxide, nitrous oxide, or hydroxylamine as potential electron acceptors. This reported lack of coupling with nitrous oxide reduction may result during preparation of the extracts from physical disruption of the sensitive coupling of phosphorylation to the electron transport chain. Otherwise, the observation would be inconsistent with the ability of *M. denitrificans* to grow anaerobically in complex media supplied with nitrous oxide as the terminal oxidant (244).

Particles recovered from *M. denitrificans* and *P. denitrificans* cells that were ruptured by alternating freeze-thaw cycles (222, 223), or those from *M. denitrificans* cells broken by lysozyme treatment and dilution (145), couple phosphorylation to transfer between NADH and cytochrome *c* (possibly in conjunction with the oxidation of NADH by Q_0 [135]). Phosphorylation effected by the particles derived from the osmotically broken cells is coupled to reduction of oxygen or nitrate, but not nitrite. In fact, the particles are freed of a nitrite-reducing system in preparation. Average P-NO_3^- ratios of 0.9 and 0.06 are obtained with NADH and succinate, respectively, serving as electron donors. Addition of ADP and P_i increases rates of electron transport in the particles. Significantly, rates of phosphorylation depend on the simultaneous presence of both electron donor and acceptor.

KCN, DNP, arsenate, and amytal inhibit phosphorylation related to nitrate reduction, but carbon monoxide does not (145, 222, 237).

In addition to assaying esterification of radioactively labeled phosphate accompanying reduction of nitrate, indirect methods of estimating ATP generation have been used as well. Hadjipetrou and Stouthamer (122) employ the relationship between ATP production and cell yield (10.5 g, dry weight, per mol of ATP produced) inferred from the values obtained for various bacteria by Bauchop and Elsden (15) to calculate the probable relationship between nitrate reduction and ATP generation in *E. aerogenes*. They note that the yields in grams (dry weight) per mole of glucose and mannitol fermented in minimal media are 26.1 and 21.8, respectively. Anaerobic growth at the concurrent expense of nitrate respiration and fermentation in identical media raises the yields to 45.5 and 50.6. Deducing ATP production from the molar growth yields previously established for this organism, it may then be calculated that approximately 3 mol of ATP are produced per mole of nitrate reduced to nitrite. The electron flow that reduces nitrate to nitrite provides all the nonfermentation-related phosphorylation achieved under these conditions. Assimilatory reduction of nitrite to ammonia yields no ATP.

ECOLOGICAL STUDIES

Nitrate Fluxes in Soil

Along with losses incurred by the relatively easy washout mobility of the negatively charged nitrate ion through soil and into watersheds, denitrification has long been recognized as an agriculturally undesirable source of loss of nitrogen from soil (297). Measurement of the quantities of either nitrogen or nitrous oxide (326) liberated by nitrate reduction has been employed for several decades to estimate the losses from different types of soils, and many of the factors that contribute to loss by either microbial or chemical (221, 324, 348) activity have been determined. Thus, rates of release of nitrogen are increased by the following agen-

cies: (i) increasing soil moisture to the point that precludes oxygen penetration (61, 249, 273, 325, 366), (ii) slight alkalinity (297), (iii) moderate to elevated temperature (192, 220), although denitrification occurs in tundra even at 4 to 8 C (185), (iv) respiratory consumption of oxygen by roots and soil microorganisms (22, 325, 326), and (v) availability of oxidizable organic compounds to serve as electron donors (205, 324). In contrast, rates of release of nitrogen from soil are diminished by dryness (220), aeration (111), adding straw (115), and soil acidity (180, 297). Dramatic increases in denitrification in previously arid desert soil accompany moistening and the addition of organic material (192). The oxygen content of soil need not fall below a measurable quantity for denitrification to occur in pockets where depletion may be more extensive than instruments will perceive; but loss from reduction is most rapid when complete anoxia is reached (111, 220).

Careful management of soil conditions minimizes release of nitrogen by denitrification. For example, application of graded quantities of nitrate to a crop at intervals throughout the active growing season minimizes loss, whereas large-scale treatment of soil with nitrate before planting results in significant loss (328, 383). Even during active growth of a crop such as wheat, application of nitrate to soils poor in potassium provides the right conditions for extensive denitrification, for the plants that are physiologically slowed down by potassium starvation compete only poorly with the soil bacteria for nitrate (362). The flooding of soils in preparation for rice culture soon after spring thawing provides conditions that favor denitrification at the expense of the residual quantities of organic matter that have not been degraded during the colder months of winter. Flooding of the soil later in the spring, after the previous season's residue of organic material has been oxidatively degraded, then diminishes loss of nitrogen (134).

Chemical treatment of soils may protect against nitrate losses in some degree. Application of antibiotics transiently suppresses growth of denitrifiers (93) as well as other bacteria. Furthermore, application of chlorate as a herbicide has a concomitantly inhibitory influence on the denitrifying population of soil as a consequence of the ability of the dissimilatory nitrate reductases, which the bacteria produce, to reduce chlorate to chlorite in toxic quantities (151). This inhibitory effect is transient as well. Supplementation of anaerobic soil with sulfide decreases loss of nitrogen and stimulates assimilatory reduction of nitrate to ammonia, but elevated sulfide concentrations will suppress any sort of nitrate reduction (219). At 20 mmol/g of soil the NH_3-N_2 ratio is optimal.

Denitrification simply impoverishes soil of a nutrient; but in addition to the loss of nutrient, nitrate washout or drain-off effected by rain or irrigation water can result in pollution of the environment as well. If the accumulation of this anion causes a significant increase in the nitrate concentration in drinking water in areas drawing from the watershed, a particular hazard is created for the young consumer. As much as 55 to 60% of the nitrate in water that runs off farmlands may originate from applied fertilizers (170), although the assays used to arrive at this figure have been questioned (127). As mentioned before, judicious application of fertilizers helps prevent such accumulation, but other sources of nitrate may also represent hazards. For example, cutting away covering vegetation contributes to upsurges in populations of *Nitrosomonas* and *Nitrobacter* species in the soil that is thus laid bare and increases the nitrate content of the soil and its watershed (312). In addition, for a time after peat beds are drained in preparation for conversion of the land to cultivation, nitrate accumulates in the peat and reaches agriculturally undesirable levels. But this can be managed. It is found that after a short time much of the organic matter is degraded by resident microorganisms, and oxygen is consumed in the process. Degradation or the organic material continues and extensive denitrification is initiated as anoxia is approached. This decreases the nitrate content to a degree that makes the formerly toxic soil acceptable for agricultural use (7).

Other examples of good management can be cited. The nitrate content of the output of sewage treatment operations may be diminished by using the effluent for irrigating soil plots planted with non-edible crops. Addition of oxidizable organic material may be necessary to ensure reduction in the soil of any nitrate not assimilated by the crop plants (180). Finally, placement of cattle feed lots on level, absorbent (rather than sloping, nonabsorbent) land ensures that the significant amounts of nitrate formed by nitrification of the ammoniac products of excreta will be removed by denitrification in the soil beneath the animals rather than washed into the lakes, ponds, and streams in the surrounding area (73).

Engineering Practices for Diminishing Nitrate Pollution

Effluents that emerge from sewage treatment plants or waters that drain from agricultural

land with nitrate concentrations great enough to encourage unwanted growth of water plants or to constitute a hazard in drinking water can be denitrified by holding in anaerobic ponds or deep basins (13, 165, 217) or passage through an anaerobic filter before final release (346, 349). The bacteria in these systems are influenced by holding temperature as well as oxygen content. As expected, they function slowly at 5 C, but more rapidly at higher temperatures. Natural systems behave much like control denitrifying cultures of *P. denitrificans* (54). Any number of organic compounds will serve as electron donors. Sucrose and lactose have been employed as the electron donors for the denitrification of groundwater before it is used as drinking water (165), but methanol is the electron donor of choice for promoting denitrification in water (204). Its oxidation by nitrate yields only nitrogen, carbon dioxide, water and cells. A 2:1 to 3:1 ratio of methanol to nitrate is indicated by model experiments, and greater ratios may leave residues that contribute to unwanted increases in biochemical oxygen demand (59). Waste water from soil that is poor in organic matter may be stripped of nitrate by addition of sulfur as an electron donor that will promote the denitrifying growth of *T. denitrificans* (196).

Once an effective microbial population is established in a filter that must be shut down for cleaning by backwash, retention of a portion of the old filter for use an an inoculum is a useful procedure which permits reestablishment of the denitrifying efficiency of the filter more rapidly than rebuilding an effective population by natural selection (179). Residence time required for removal of nitrate by the organisms in any filter or pond system depends on nitrate and carbon source loads, pH, and temperature; but complete mixing is necessary for conversion to gaseous products irrespective of other conditions (213).

Even in materials such as poultry wastewater that contain high concentrations of ammonia, denitrification can be utilized for removing the pollution potential. The wastes are first aerated vigorously to convert ammonia to nitrate and then are held under anaerobic conditions to permit denitrification to occur (218). More than one cycle is usually required when no exogenous source of electrons is provided (275).

Phenomena Observed in Natural Waters

In both fresh and salt water, as in culture, denitrification is favored by lowered oxygen tension and availability of oxidizable organic matter (143). Unlike denitrification in soil, dissimilatory reduction of nitrate in freshwater lakes releases only nitrogen in significant quantity, and seldom is any nitric or nitrous oxide detected in the usual experiment (36, 101). Large-volume analyses are required to reveal nitrous oxide in marine water and the atmosphere above it. Kinetic studies show that the nitrous oxide is slowly decomposed (147). In lake water, as in culture, nitrite accumulates prior to active gas production, although a certain amount of nitrogen is liberated during the nitrite-accumulation period. Where there are rich organic sediments, approximately two-thirds of the nitrate nitrogen flowing or seeping in is lost by denitrification and slightly more than one-third is assimilated (35, 154).

As anticipated from their behavior when isolated, marine algae in the open sea most actively assimilate nitrate while photosynthesis is proceeding most rapidly (100). These algae adapt their capacities to their needs. Kinetic studies show that the phytoplankton in oligotrophic marine waters are characteristically able to take up nitrate much more rapidly than the residents of eutrophic zones (194). The ability to couple nitrate utilization rapidly with response to light has some value in predicting the competitive edge one alga may have over another when they occur together in various zones of the ocean (78). Ammonia is, of course, the preferred source of nitrogen for the algae, but nitrate and nitrite are utilized (29, 380). One study shows that nitrate provides 8.3% of the nitrogen supply for phytoplankton in subtropical marine waters and 39.5% in temperate waters (71). Nitrogen fixation and ammonia assimilation supply the greater quantity, but there are circumstances under which nitrate utilization predominates. The phytoplankton in a discontinuity layer in an otherwise euphotic zone of subtropical water use nitrate as their principal source of nitrogen (103). It thus seems likely that the contribution of algal assimilation of nitrate nitrogen to the total nitrogen budget in marine waters will be difficult to estimate. There are yet no estimates of the contributions attributable to bacterial assimilation.

In natural waters, however, dissimilatory nitrate reduction is attributable to bacteria alone and is extensive. Denitrification occurs in various types of marine waters low in oxygen content (29, 97, 99). Nitrate-rich waters that pass into the organic rich sediments of the continental shelf are stripped of nitrate by denitrification that is particularly intense at the sediment-water interface (102). Activity diminishes deeper into the sediments. Not all of the dissimilatory reduction results in immediate gas release. Nitrite accumulates in oxygen mini-

mum layers of the sea as a consequence principally of nitrate reduction rather than oxidation of ammonia (30, 381). A certain amount of nitrogen is released during the accumulation of nitrite, but liberation becomes most rapid after nitrate is depleted (98). In the sediments in bays or in water as deep as 40 m, it is estimated that 50% of the organic matter degraded is oxidized by nitrate-reducing bacteria (287).

CONCLUDING REMARKS

Strangely, it is the assimilatory nitrate reductases of algae and fungi, but the dissimilatory nitrate reductase of bacteria, that contain molybdenum. Taken together, the interchangeable nature of the molybdenum-containing subunits of the fungal and bacterial (and other) enzymes and the observation that each reduces chlorate suggest that protein can bind molybdenum in only a limited number of configurations to achieve an electron-transporting form. A determination of the structure of the active binding sites may shed some light on the mechanisms of electron transport mediated by molybdenum. A heavy metal may also be involved in the reduction carried out by bacterial assimilatory nitrate reductase (which does not reduce chlorate), but molybdenum has not been implicated in this reaction. If a different mechanism is involved, it should be elucidated. Until oxygen-sensitive and insensitive B-type enzymes from bacteria are purified and characterized, no clear explanation for the differences between this type of reduction and that carried out by molybdenum-rich enzymes will be possible.

Clarification of the mechanisms regulating synthesis and specification of the steps involved in the functioning of assimilatory nitrite reductase are needed as well. Production is initiated in algae and fungi by the presence of nitrite and/or a nutritive void created by the absence of ammonia. There is still a question as to whether or not either endogenous or exogenous nitrite must be present for synthesis to be initiated. Requirements for synthesis of assimilatory nitrite reductase in bacteria are further complicated by the influence of oxygen on some but not all and also by possible confusion between nitrite- and sulfite-reducing mechanisms. The notion that the reduction of nitrite to ammonia is designed as a detoxification measure is not encountered in studies of other organisms. Nothing is known of control and little is known of the intermediate electron transport events in the nitrite reduction carried out by obligately anaerobic bacteria. Like algae, they engage ferredoxin early in the operation of the electron transfer chain, but none of the cofactors or metals participating in the terminal events (reduction of nitrite, possibly nitric oxide, and hydroxylamine) is known. Isolation of mutants that produce only partially effective nitrite reductases may provide the experimental materials needed for examining questions raised by the work carried out to date.

Comparison of the molecular weights of nitrate and nitrite reductases is informative only if the degree of complexity of the materials assayed is specified (Table 3). When the entire electron transport chain is included in the

TABLE 3. *Comparison of molecular weights of nitrate and nitrite reductases from various microorganisms*

Reductase	Organism and ref. no.								
	Aspergillus nidulans[a] (64)	*Chorella fusca*[a] (394)	*Dunaliella tertiolecta*[a] (182)	*Enterobacter aerogenes*[b] (368)	*Micrococcus denitrificans*[b] (84)	*Achromobacter cycloclastes*[b] (140)	*Alcaligenes faecalis*[b] (139)	*Dunaliella tertiolecta*[a] (106)	*Pseudomonas perfectomarinus*[b] (51)
Nitrate									
NADH linked		500,000	500,000						
NADPH linked	197,000								
Reduced dye dependent				400,000 (dimer) 200,000 (monomer)	160,000				
Nitrite									
Reduced ferredoxin linked or reduced PMS linked		63,000				69,000	90,000	70,000	
NADH linked									200,000

[a] Assimilatory.
[b] Dissimilatory.

aggregate weighed, no significant information about the protein catalyzing nitrate or nitrite reduction is provided. Comparisons do make obvious the greater complexity of nitrate than nitrite reductases, however.

Characterization of genetic and other regulating factors, the structure, and many functions of dissimilatory nitrate reductase has proceeded well. Among the further dissimilatory, denitrifying enzymes, the most significant progress has been achieved from studies of nitrite reductase. But, understanding at an acceptable depth can only come when the component enzymes and cofactors that participate in each of the intermediate steps are separated and characterized individually. Attempting to sort out specific contributions of various cofactors to the reduction of nitrite, nitric oxide, and nitrous oxide in complex enzyme mixtures is likely to remain confusing. Moreover, the convention of designating the *c*-type cytochromes involved in denitrification by the absorption maximum of their α bands must be replaced by acknowledgment of more distinctive properties. All those observed to date have α-band absorption maxima near 550 nm despite their disparate functions.

The importance of estimates of the contributions of assimilatory nitrate reduction and denitrification rates to establishment of ecosystem nitrogen budgets has been increasingly realized by workers in the field. As is true with many ecological problems, however, the development of sampling and assay methods lags behind need. Gas chromatography has made enzyme studies possible and offers promise for studies of denitrification in nature; but for some time to come, cross-checking of results obtained in the environment by ^{15}N assays may be desirable until the reliability of the gas chromatographic procedures used can be established.

ACKNOWLEDGMENTS

I am grateful for the aid provided by the University of Georgia Computer Center's Information Retrieval Service.

My appreciation is extended as well to Daniel Vapnek and D. V. Dervartanian for discussion and criticism.

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